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THE ROLE OF CALCIUM FLUX

AND ITS RELATIONSHIP TO VITAMIN D RESPONSE

IN AN <u>IN</u> <u>VITRO</u> MODEL OF CHONDROGENESIS

A

THESIS

Presented to the Faculty of

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By

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San Antonio, Texas

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THE ROLE OF CALCIUM FLUX AND ITS RELATIONSHIP TO VITAMIN D RESPONSE IN AN IN VITRO MODEL OF CHONDROGENESIS

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DEDICATION

With great pleasure and pride, I dedicate this thesis to my wife and best friend, Susan. Her unending love, understanding and patience have given real meaning to my life. I also dedicate this to our son, Jonathan, whose presence and smile keep all other things in perspective.

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THE ROLE OF CALCIUM FLUX

AND ITS RELATIONSHIP TO VITAMIN D RESPONSE

IN AN IN VITRO MODEL OF CHONDROGENESIS

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Supervising Professor: Barbara D. Boyan

It is well established that Vitamin D metabolites affect proliferation, differentiation and maturation of cartilage cells.   Previous studies have shown that growth zone chondrocytes respond primarily to $1,25-(OH)_2D_3$ whereas resting zone chondrocytes respond primarily to $24,25-(OH)_2D_3$.   This study used the ionophore, A23187, to examine the hypothesis that the effects of vitamin D on cartilage cells are mediated by changes in calcium flux.   Confluent, fourth passage cultures of growth zone and resting zone chondrocytes from the costochondral

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cartilage of 125 gram rats were used as the model system since these cells retain their differential response to the vitamin D metabolites. To examine whether calcium content might play a role in the mechanism of hormone action, cells were incubated with A23187 (0, 0.01, 0.05, 0.1, 1.0 and 10 uM) for 3, 6, 12, 15 or 30 minutes. Alkaline phosphatase (ALPase) and phospholipase A_2 (PA_2) are two enzymes shown previously to be regulated by $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$. The specific activity of these enzymes was measured in the cell layer and in isolated plasma membranes and matrix vesicles. To determine whether the effect of ionophore was comparable to that of the vitamin D metabolites, this study also compared the effects of $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$.

In a second set of experiments, the effects of A23187, $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ on $^{45}Ca^{2+}$ flux of the cells were determined. In these experiments, resting zone chondrocytes and growth zone chondrocytes were incubated with A23187 for 30 minutes. Resting zone chondrocytes were also exposed to $24,25-(OH)_2D_3$ for up to 60 minutes and growth zone chondrocytes were exposed to $1,25-(OH)_2D_3$ for 1-60 minutes. Influx was measured as incorporation of $^{45}Ca^{2+}$. Efflux was measured as release of $^{45}Ca^{2+}$ from pre-labelled cultures into fresh media.

Resting zone chondrocytes and growth zone chondrocytes responded to A23187 in a comparable manner. Alkaline phosphatase specific activity was inhibited at 0.1 uM A23187 in resting zone chondrocytes and 0.1 and 1 uM in growth zone chondrocytes. This inhibition correlated with the increase in

$^{45}Ca^{2+}$ content. Both the plasma membrane and matrix vesicle enzyme activities were inhibited. There was no statistically significant effect of ionophore on matrix vesicle or plasma membrane phospholipase A_2 in either cell type. In contrast, alkaline phosphatase activity was stimulated when growth zone chondrocytes were incubated with $1,25-(OH)_2D_3$ or in resting zone chondrocytes which were incubated with $24,25-(OH)_2D_3$. Phospholipase A_2 activity was differentially affected depending on the metabolite used and the cell examined. Addition of ionophore to cultures preincubated with $1,25-(OH)_2D_3$ or $24,25-(OH)_2D_3$ blocked the stimulation of alkaline phosphatase by vitamin D in a dose-dependent manner. These results suggest that the effects of vitamin D on membrane enzyme activity involve mechanisms other than just $^{45}Ca^{2+}$ influx per se.

The effect of A23187 on $^{45}Ca^{2+}$ flux was different from that induced by either $1,25-(OH)_2D_3$ or $24,25-(OH)_2D_3$. In unstimulated (control) cells, the pattern of $^{45}Ca^{2+}$ influx was different in the two chondrocyte populations; however, the pattern of efflux was comparable. A23187 induced a rapid influx of $^{45}Ca^{2+}$ in both types of chondrocytes which peaked by 3 minutes and was over by 6 minutes. Influx was greatest in the growth zone chondrocytes. The two cell populations exhibited a cell-dependent response to the vitamin D metabolites. In addition, the effects of hormone were distinctly different from those of the ionophore. Addition of 10^{-8} to 10^{-7} M $1,25-(OH)_2D_3$ to growth zone chondrocyte cultures resulted in a dose dependent increase in $^{45}Ca^{2+}$ influx after 15 minutes. Efflux was

stimulated by these concentrations of hormone throughout the incubation period. Addition of 10^{-6} to 10^{-7} M 24,25-(OH)$_2$D$_3$ to resting zone chondrocytes resulted in an inhibition in ion efflux between 3-6 minutes, with no effect on influx during this period. A large efflux between 6-15 minutes resulted in a return to control values. ^{45}Ca^{2+} influx was inhibited by these concentrations of hormone from 15-30 minutes. These studies demonstrate that changes in calcium influx and efflux are metabolite-specific and may be a mechanism by which vitamin D metabolites directly regulate chondrocytes in culture.

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I. INTRODUCTION AND LITERATURE REVIEW

A. Endochondral Ossification

Osteoinduction is an important concept in the understanding of fracture healing, wound management and bone grafting. Initial fracture healing often involves cells with a chondrogenic potential (Simmons, 1985). Cartilage can be stimulated to differentiate, calcify and support subsequent bone formation (Urist and McLean, 1953). This process can be induced in heterotopic and orthotopic mesenchymal tissues which would otherwise not form bone by proteins present in bone termed Bone Morphogenetic Protein (BMP) (Urist and Mikulski, 1979). BMP-induced bone repair appears to proceed by a mechanism comparable to endochondral ossification. The calcification of cartilage initiated by the chondrocyte is a very important step in this healing process. Membrane-bound matrix vesicles formed by the chondrocytes have been identified as a key factor in cartilage calcification (Anderson, 1969).

To understand bone wound healing we must first understand the role of cartilage in endochondral ossification and its regulation. To study this, an in vitro model has been developed using chondrocytes cultured from the costochondral cartilage of rats to examine matrix vesicle production and cell regulation (Boyan et al., 1988b). These experiments established two populations of chondrocytes in different stages of differentiation and is being used to study the effects of vitamin D [specifically the $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ active metabolites of vitamin D] on the plasmalemma and matrix

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vesicle membranes of growth zone and resting zone chondrocytes (Boyan et al., 1988a). Differences in stimulation of chondrocytes and matrix vesicles by the different metabolites of vitamin D suggest that the activation is cell-specific and metabolite-specific. Thus, response to vitamin D metabolites can be used as a marker of cell differentiation in vitro.

The biochemical basis for the mechanism of vitamin D action in chondrocytes has not been elucidated. It has been shown in many studies (Ornoy and Langer, 1978; Boyan et al., 1988b) that resting zone and growth zone chondrocytes are morphologically different and that there is an increase in alkaline phosphatase (ALPase) specific activity as the cells differentiate from the resting zone phenotype to the growth zone (hypertrophic) phenotype. Boskey (1981) described different stages of chondrocyte differentiation and activity. Resting zone cartilage is composed of cells dispersed in a proteoglycan rich matrix. These cells align into columns and proliferate. After this proliferative stage, the cells produce extracellular matrix vesicles (Anderson, 1969). Subsequently, the chondrocytes hypertrophy and calcify their extracellular matrix with initial calcification occurring in the matrix vesicles (Brighton and Hunt, 1976). Studies using isolated matrix vesicle preparations (Schwartz et al., 1988a) indicate that most of the increase in alkaline phosphatase activity associated with cartilage calcification is localized to the matrix vesicles.

B.   <u>Vitamin D$_3$</u>

Vitamin D$_3$, from sunlight conversion of 7-dehydrocholesterol in the skin (Guyton, 1976) or from dietary sources, is hydroxylated to 25(OH)D$_3$ in the liver, followed by hydroxylation to 1,25-(OH)$_2$D$_3$ (Haussler, 1986) and 24,25-(OH)$_2$D$_3$ in the kidney. A multitude of disease states have been related to vitamin D including rickets, osteomalacia, osteoporosis, hyperparathyroidism, hypoparathyroidism, cirrhosis, thyroid carcinoma, chronic renal disease, sarcoidosis, malabsorption syndrome and tropical sprue (Norman, 1980).

One action of vitamin D$_3$ metabolites is to change the phospholipid composition of the plasma membrane (Boskey and Weintroub, 1986; Boyan et al., 1988a; Putkey et al., 1986), resulting in fluidity and permeability modifications. According to this hypothesis, calcium transport <u>may</u> occur without <u>de</u> <u>novo</u> mRNA and protein synthesis by changing phospholipase A$_2$ activity (Schwartz and Boyan, 1988). A similar mechanism has been postulated for other cell mediators as well. For instance, Chang et al. (1986) showed that interleukin-1 increased phospholipase A$_2$ without an increase in intracellular protein. They postulated that interleukin-1 activated existing phospholipase A$_2$ rather than by inducing new enzyme synthesis. Calcium ionophores appear to have a comparable effect on cells in culture (DeLuca and Schnoes, 1976).

1.   <u>1,25-(OH)$_2$D$_3$</u>. Recent investigators have reported a multiplicity of actions of 1,25-(OH)$_2$D$_3$, including a rapid direct action in altering intestinal membrane transport of Ca^{2+}

prior to the synthesis of specific transport proteins (Nemere
and Norman, 1986; Wasserman and Fullmer, 1983; Fullmer et al.,
1984; Yoshimoto and Norman, 1986; Wasserman and Taylor, 1969).
This rapid effect has been observed in bone forming cells as
well. In mouse osteoblasts, $1,25-(OH)_2D_3$ induces immediate
transient increases in cytosolic Ca^{2+} (Lieberherr, 1987). In
addition to its effects on mineral transport and mineralization,
$1,25-(OH)_2D_3$ has other biologic effects including affecting
sterol levels in skin (Esvelt et al., 1980), stimulating
macrophage differentiation (Mangelsdorf et al., 1984), and
influencing the secretion of other hormones [e.g. parathyroid
hormone (Dietel et al., 1979), prolactin (Wark and Tashjian,
1982), and insulin (Kadowaki and Norman, 1984)]. $1,25-(OH)_2D_3$
has been shown by Nemere et al. (1987) to regulate tubulin
expression in normal chick duodenum. In vivo administration of
$1,25-(OH)_2D_3$ increased cellular tubulin resulting in decreased
mRNA levels.

Reichel et al. (1987b) suggested that LPS-stimulated
macrophages could locally produce $1,25-(OH)_2D_3$ which may
influence the function of lymphocytes and synthesis of
lymphokines (Tsoukas et al., 1984). Thus, $1,25-(OH)_2D_3$ may play
an immunoregulatory role. In a later study, Reichel et al.
(1987a) postulated that $1,25-(OH)_2D_3$ produced by pulmonary
alveolar macrophages may be involved in alveolar space signals
between macrophages and lymphocytes to down-regulate the immune
response. Also dexamethasone, a steroid with anti-inflammatory
properties decreased $1,25-(OH)_2D_3$ in normal and sarcoid

pulmonary alveolar macrophages. Lipopolysaccharide (LPS), a potent inflammatory agent, increased synthesis of $1,25-(OH)_2D_3$ (Reichel et al., 1987a). Evidence of an immune regulatory function was also presented by Reichel et al. (1987c) in a study demonstrating $1,25-(OH)_2D_3$ production by human T-lymphotropic virus-I (HTLV-I) infected lymphocytes which may result in hypercalcemia in lymphoma/leukemia patients infected by HTLV-I. Tobler et al. (1987) showed that $1,25-(OH)_2D_3$ inhibited synthesis of granulocyte-macrophage colony-stimulating factor (GM-CSF) in normal human peripheral blood lymphocytes. They concluded that $1,25-(OH)_2D_3$ may be important in hematopoiesis physiology.

2. $\underline{24,25-(OH)_2D_3}$. Specific nuclear and cytoplasmic binding of $24,25-(OH)_2D_3$ has been shown (Somjen et al., 1982) in mesenchymal cells and chondrocytes indicating that they have receptors for this metabolite. Wilhelm et al. (1986) showed that the binding domain/receptor for $24,25-(OH)_2D_3$ on chick intestinal mucosa is independent of the $1,25-(OH)_2D_3$ receptor suggesting that these two metabolites have distinct effects on cells. Several investigators have shown differential effects from those of $1,25-(OH)_2D_3$ (Lieberherr et al., 1984; Schwartz and Boyan, 1988; Harmand et al., 1984). Based on enhancement of protein synthesis and [^{3}H]-thymidine incorporation into DNA in embryonic chick skeletal mesenchyme undergoing chondrogenesis, Binderman and Somjen (1984) suggested that $24,25-(OH)_2D_3$ was important in epiphyseal cartilage development and bone formation.

Fine et al. (1985) localized $24,25\text{-(OH)}_2D_3$ to the cytoplasm and nucleus of all cell layers of the epiphysis except the hypertrophic cartilage zone. After 15 minutes, radioactivity marking the $24,25\text{-(OH)}_2D_3$ was seen mainly in the cell membrane and cytoplasm. At 60 minutes, radioactivity was also prominent in the nuclei.

Both $1,25\text{-(OH)}_2D_3$ and $24,25\text{-(OH)}_2D_3$ appear to be essential for bone formation in fracture repair. Experiments using chicks as a model (Dekel et al., 1983) demonstrate that vitamin D_3 and its metabolites vary in their ability to increase the strength of callus repair in the following decreasing order: $24,25\text{-(OH)}_2D_3$ plus $1,25\text{-(OH)}_2D_3$ > cholecalciferol > $1,25\text{-(OH)}_2D_3$ > $24,25\text{-(OH)}_2D_3$. Increased levels of $24,25\text{-(OH)}_2D_3$ were found in calluses and in the epiphyses of the fractured tibia in chicks. Greater demands for $24,25\text{-(OH)}_2D_3$ during long bone growth could explain the higher levels in the tibia (Lidor et al., 1987).

3. <u>Mode of Action</u>. The mode of action of vitamin D is thought to be genomic and resemble that of other steroid hormones (Narbaitz, 1983). However, the biochemical basis for this mechanism in chondrocytes is not well characterized. Recent publications have shown at least some of the effect of the hormone may be due to direct action of vitamin D_3 metabolites on both plasma membrane and matrix components (Schwartz et al., 1988a). That investigation demonstrated a differential effect of $1,25\text{-(OH)}_2D_3$ and $24,25\text{-(OH)}_2D_3$ on the specific activity of alkaline phosphatase and phospholipase A_2 on plasma membranes and matrix vesicles of growth zone and

resting zone chondrocytes in culture. Effects of these hormones were demonstrated on isolated matrix vesicle preparations, thus demonstrating the direct effect in the absence of cellular regulation mechanisms. Neither hormone had any effect on these enzymes in cultures of neonatal rat muscle mesenchymal cells which do not calcify in vivo (Schwartz et al., 1988a).

Recently, an in vitro model using chondrocytes cultured from rat costochondral cartilage was developed in order to characterize chondrocytes at various stages of differentiation as well as to examine their regulation, particularly by vitamin D_3 (Boyan et al., 1988a). This model has been used to study the effects of $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ on the plasma membranes and matrix vesicles isolated from cultures of both resting zone and growth zone chondrocytes. Resting zone chondrocytes respond primarily to $24,25-(OH)_2D_3$. Matrix vesicle alkaline phosphatase is stimulated (Schwartz et al., 1988a), phospholipase A_2 activity is inhibited (Schwartz and Boyan, 1988), and production of noncollagenous proteins is stimulated (Schwartz et al., 1989) in a dose dependent manner. In contrast, growth zone cells respond primarily to $1,25-(OH)_2D_3$. Alkaline phosphatase activity (Schwartz et al., 1988a), phospholipase A_2 activity (Schwartz and Boyan, 1988) and collagen production (Schwartz et al., 1989) are all stimulated by $1,25-(OH)_2D_3$ in these cells. The results of the studies indicate that response to these vitamin D_3 metabolites can be used as a marker of cell differentiation in vitro (Boyan et al., 1988b).

C.   The Calcium Messenger System

It is possible that many of the effects of vitamin D on cartilage cells can be attributed to changes in intracellular Ca^{2+} concentration (Tanaka and DeLuca, 1971). In the intestinal epithelium, intracellular Ca^{2+} is increased rapidly in response to $1,25-(OH)_2D_3$ whereas increased synthesis of alkaline phosphatase and other vitamin D-dependent proteins occurs much later (Bikle et al., 1978). Intracellular Ca^{2+} is also increased by high doses of $24,25-(OH)_2D_3$.

Studies in our laboratory using isolated matrix vesicle preparations indicate that at least some of the response of chondrocytes to $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ is independent of new gene transcription or translation and may be due to direct effects of hormone on the plasma membrane and matrix vesicles (Schwartz et al., 1988a). Vitamin D metabolites change the phospholipid composition of the membrane in both intestinal cells and cultured chondrocytes (Boyan et al., 1988a; Rasmussen et al., 1982; Putkey et al., 1986; Nemere and Norman, 1986) with resultant fluidity and permeability modifications postulated to be a mechanism by which the metabolites act. For example, in the intestine, $1,25-(OH)_2D_3$ stimulates calcium transport without de novo mRNA and protein synthesis by changing the lipid composition of the membrane (Tanaka and DeLuca, 1971; Bikle et al., 1978; Levy et al., 1987).

There are two pools of calcium which contribute to changes in cytoplasmic Ca^{2+} concentration. Both may be involved in Ca^{2+}

flux due to the action of vitamin D metabolites. These two pools are:

1.   <u>Extracellular calcium</u>. In nerve cells, influx of Ca^{2+} from the extracellular pool leads to membrane depolarization. The plasma membrane is the site of action, calmodulin is the intracellular calcium receptor protein and Ca^{2+} cycles across the membrane to initiate and terminate the Ca^{2+} signals (Rasmussen, 1986a).

2.   <u>Intracellular calcium</u>. In skeletal muscle, release of Ca^{2+} from the sarcoplasmic reticulum results in increased cytosolic Ca^{2+} which is detected by troponin C, a specific Ca^{2+} receptor protein.   This binding eventually results in a contractile response (Rasmussen, 1986a).

These two pools may be interrelated. Cardiac muscle is an example of this (Rasmussen, 1986a).   A small influx of extracellular Ca^{2+} due to plasma membrane depolarization leads to release of Ca^{2+} from the sarcoplasmic reticulum.   In these "excitable" cells, the Ca^{2+} response is fast and usually repetitive.

3.   <u>Calcium as a second messenger</u>.   In "nonexcitable" cells, Ca^{2+} also serves as an intracellular messenger. Hydrolysis of the phospholipid phosphatidylinositol-4-5-bisphosphate, in the cell membrane produces inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DG) which act as intracellular messengers (Rasmussen, 1986b).   This change in membrane phospholipids initiates a cascade of events including calcium mobilization, activation of protein kinase C (Nishizuka,

1984), and arachidonic acid release (resulting in synthesis of prostaglandins, thromboxanes and leukotrienes) (Berridge, 1981, 1984).

Release of Ca^{2+} from an intracellular pool (such as endoplasmic reticulum or mitochondria) is induced by inositol 1,4,5-trisphosphate (IP_3) generated at the plasma membrane (Rasmussen, 1986b). When Ca^{2+} concentration rises above a certain threshold in the membrane, four Ca^{2+} ions bind to each molecule of calmodulin, a specific calcium binding protein which is activated by its binding with Ca^{2+}. Activated calmodulin in turn activates adenylate cyclase which then converts adenosine triphosphate (ATP) into the intracellular messenger, cyclic adenosine monophosphate (c-AMP). Having performed its intracellular messenger function, c-AMP is degraded to 5'-adenosine monophosphate (5'-AMP) by phosphodiesterase which is activated by active calmodulin in the cytoplasm. Again, the calmodulin activation depends on binding with Ca^{2+} (Cheung, 1980).

Phospholipase A_2 (PA_2) is a calcium dependent enzyme which catalyzes the release of arachidonic acid from phosphatidycholine, phosphatidylethanolamine and phosphatidylinositol. This pathway of arachidonic acid release requires much higher levels of calcium than release catalyzed by diacylglycerol (Billah et al., 1980) and may only occur at supramaximal stimulation (Berridge, 1984).

4. __Calcium efflux__. After intracellular increases of Ca^{2+}, either through ion channels or by membrane-receptor

interactions, cellular homeostasis must be maintained by uptake of Ca^{2+} into intracellular organelles or by efflux from the cell. This efflux is maintained by at least two ATP-dependent mechanisms. Ca^{2+} is pumped against the extracellular gradients which are 5,000 to 10,000 times the intracellular concentration (partially aided by low cell membrane permeability). One of these is the $Ca^{2+}/2H^+$ ATPase-dependent pump (Schatzman, 1975) and the other is the sodium-potassium ATPase-dependent pump (Blaustein, 1974).

Treatment with 1,25-$(OH)_2D_3$ of chondrocytes from rachitic chick epiphyseal growth plates reduced intracellular Ca^{2+} concentrations. This decrease correlated with increased activity of Ca^{2+}-ATPase (Lidor and Edelstein, 1987).

5. <u>Temporal calcium response</u>. Studies in systems with sustained responses such as angiotensin II-mediated aldosterone secretion, glucose-induced insulin secretion, and smooth muscle contraction, have shown two distinct roles for the branches of the calcium messenger system. Short term responses are regulated by the calmodulin branch, and the C-kinase branch is involved in the sustained phase (Rasmussen, 1986b). Studies on angiotensin II have shown rapid depletion of Ca^{2+} in the endoplasmic reticulun and removal of this Ca^{2+} by pumping out of the cell. The Ca^{2+} concentration returns to basal values in 3-5 minutes (Rasmussen, 1986a, 1986b). Silver and Stull (1982) reported an IP_3-induced release of Ca^{2+} which returned to basal values within minutes in tracheal smooth muscle. Studies on the response of chick embryo myoblasts to phorbol 12-myristate 13-

acetate (PMA) indicate that this phorbol ester increases Ca^{2+} influx and efflux (Schimmel and Hallam, 1980).

$1,25-(OH)_2D_3$ also induces rapid changes in Ca^{++} transport (Yoshimoto et al., 1986). There appear to be two response mechanisms in the chick duodenum: "the classic steroid receptor genome activation system and the second is the perfused intestine membrane response system" (Yoshimoto and Norman, 1986). The perfused system responds to lower doses of $1,25-(OH)_2D_3$.

The time course of vitamin D action on chondrocyte activity has not yet been established. Evidence exists that some effects of vitamin D may occur as early as three hours after cell exposure to hormone (Tanaka and DeLuca, 1971). Dziak and Brand (1974b) showed that calcium influx in bone cells isolated from fetal rat calvaria began to plateau in 30-60 minutes as measured by $^{45}Ca^{2+}$ uptake. A review on Ca^{2+} transport in the adrenal glomerulosa presented data showing that Ca^{2+} influx occurs rapidly and then plateaus; this is followed by a compensatory Ca^{2+} efflux which maintains cellular calcium homeostasis (Rasmussen, 1986a, 1986b). These effects occur in approximately the 1-5 minute time frame.

6. <u>Ionophores</u>. Ionophores are compounds which enhance metal cation transport across lipid membranes, by combining with ions to form lipid-soluble carriers which can diffuse across the cell membrane or by forming channels in the membrane through which ions may pass (Brasseur and Ruysschaert, 1986). One such ionophore, A23187, has been used in a number of different cell

systems to increase calcium ion influx. In particular, Dziak et al. (Dziak and Brand, 1974a; Dziak and Brand, 1974b; Dziak and Stern, 1976) have used A23187 to examine the role of calcium in the hormonal regulation of bone cells.

Michell et al. (1977) have suggested that the formation of phosphatidic acid could function as an ionophore. Serhan et al. (1982) also sought evidence of "endogenous" ionophores which were products of the cell's own lipid metabolism. They concluded that phosphatidic acid (which can be generated as part of the phosphatidylinositol pathway) and cis-leukotriene B_4 (through lipoxygenation of arachidonate) can act separately or together as calcium ionophores. Serhan et al. (1982) tested A23187 in neutrophil liposomes and determined the order of potency to be A23187 > cis-leukotriene B_4 > phosphatidic acid.

D.   Study Objectives

The present study examined the hypothesis that the regulation of alkaline phosphatase and phospholipase A_2 activity by vitamin D_3 metabolites in cartilage cells is mediated by rapid changes in calcium influx. In the first set of experiments, the rapid increase in intracellular Ca^{2+}, postulated to occur in response to $1,25-(OH)_2D_3$ or $24,25-(OH)_2D_3$, was compared to that of A23187, an agent known to increase intracellular calcium. These experiments examined the effect of ionophore on alkaline phosphatase and phospholipase A_2, two enzymes previously shown to be differentially regulated by these vitamin D metabolites in chondrocyte cultures (Boyan et al., 1988a). Any effects of the ionophore were correlated with

the effects of vitamin D metabolites on the cells. Chondrocytes that were preincubated with $1,25-(OH)_2D_3$ or $24,25-(OH)_2D_3$ and then exposed to ionophore were also examined to understand whether there is a synergistic or antagonistic effect of A23187 and hormone.

In the second set of experiments, the effects of the Ca^{2+} ionophore A23187, $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ on $^{45}Ca^{2+}$ influx and efflux were examined in growth zone chondrocytes and resting zone chondrocytes derived from the costochondral junction of 125 gram rats. Influx was measured as incorporation of $^{45}Ca^{2+}$. Efflux was measured as release of $^{45}Ca^{2+}$ from pre-labelled cultures into fresh media.

CHAPTER I

ANTAGONISTIC EFFECTS OF A23187 ON $1,25-(OH)_2D_3$ AND

$24,25-(OH)_2D_3$ DEPENDENT STIMULATION OF ALKALINE

PHOSPHATASE ACTIVITY IN COSTOCHONDRAL CHONDROCYTE CULTURES

Abstract

This study used the ionophore, A23187, to examine the hypothesis that the regulation of alkaline phosphatase and phospholipase A_2 activity by vitamin D_3 metabolites in cartilage cells is mediated by rapid changes in calcium influx. Confluent, fourth passage cultures of growth zone and resting zone chondrocytes from the costochondral cartilage of 125 gram rats were incubated with A23187 (0, 0.01, 0.05, 0.1, 1.0 and 10 uM). Specific activities of alkaline phosphatase and phospholipase A_2 were measured in the cell layer and in isolated plasma membranes and matrix vesicles. There was an inhibition of alkaline phosphatase specific activity at 0.1 uM A23187 in resting zone cells and at 0.1 and 1 uM in growth zone chondrocytes. At these concentrations of ionophore, $^{45}Ca^{2+}$ content of the chondrocytes has been shown to increase. Both the plasma membrane and matrix vesicle enzyme activities were inhibited. There was no statistically significant effect of ionophore on matrix vesicle or plasma membrane phospholipase A_2 in either cell type. In contrast, alkaline phosphatase activity is stimulated when growth zone chondrocytes are incubated with $1,25-(OH)_2D_3$ and in resting zone cells which are incubated with $24,25-(OH)_2D_3$. Phospholipase A_2 activity is differentially affected depending on the metabolite used and the cell examined. Addition of ionophore to cultures preincubated with $1,25-(OH)_2D_3$ or $24,25-(OH)_2D_3$ blocked the stimulation of alkaline phosphatase by the vitamin D_3 metabolites in a dose-dependent manner. These results suggest that the effects of $1,25-(OH)_2D_3$ or $24,25-$

$(OH)_2D_3$ on membrane enzyme activity involve mechanisms other than just Ca^{2+} influx per se.

Introduction

The mode of action of Vitamin D is thought to be genomic and resemble that of other steroid hormones (Narbaitz et al., 1983). However, the biochemical basis for this mechanism in chondrocytes is not well characterized. Recent publications have shown at least some of the effect of the hormone may be due to direct action of vitamin D_3 metabolites on both plasma membrane and matrix components (Schwartz et al., 1988a).

Recently, an in vitro model using chondrocytes cultured from rat costochondral cartilage was developed in order to characterize chondrocytes at various stages of differentiation as well as to examine their regulation, particularly by vitamin D_3 (Boyan et al., 1988a). This model has been used to study the effects of $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ on the plasma membranes and matrix vesicles isolated from cultures of both resting zone and growth zone chondrocytes. Resting zone chondrocytes respond primarily to $24,25-(OH)_2D_3$. Matrix vesicle alkaline phosphatase is stimulated (Boyan et al., 1988a), phospholipase A_2 activity is inhibited (Schwartz and Boyan, 1988), and production of non-collagenous proteins is stimulated (Schwartz et al., 1989) in a dose dependent manner. In contrast, growth zone cells respond primarily to $1,25-(OH)_2D_3$. Alkaline phosphatase activity (Boyan et al., 1988a), phospholipase A_2 activity (Schwartz and Boyan, 1988) and

collagen production (Schwartz et al., 1989) are all stimulated by 1,25-$(OH)_2D_3$ in these cells. The results of the studies indicate that response to these vitamin D_3 metabolites can be used as a marker of cell differentiation _in vitro_ (Boyan et al., 1988b).

It is possible that many of the effects of vitamin D on cartilage cells can be attributed to changes in intracellular Ca^{2+} concentration (Tanaka and DeLuca, 1971). In the intestinal epithelium, intracellular Ca^{2+} is increased rapidly in response to 1,25-$(OH)_2D_3$ whereas increased synthesis of alkaline phosphatase and other vitamin D-dependent proteins occurs much later (Bikle et al., 1978). Intracellular Ca^{2+} is also increased by high doses of 24,25-$(OH)_2D_3$.

Studies in our laboratory using isolated matrix vesicle preparations indicate that at least some of the response of chondrocytes to 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ is independent of new gene transcription or translation and may be due to direct effects of hormone on the plasma membrane and matrix vesicles (Schwartz et al., 1988a). Vitamin D metabolites change the phospholipid composition of the membrane in both intestinal cells and cultured chondrocytes (Boyan et al., 1988a; Rasmussen et al., 1982; Putkey et al., 1986; Nemere and Norman, 1986) with resultant fluidity and permeability modifications postulated to be a mechanism by which the metabolites act. For example, in the intestine, 1,25-$(OH)_2D_3$ stimulates calcium transport without _de novo_ mRNA and protein synthesis by changing the lipid

composition of the membrane (Tanaka and DeLuca, 1971; Bikle et al., 1978; Levy et al., 1987).

Ionophores are compounds which enhance metal cation transport across lipid membranes, by combining with ions to form lipid-soluble carriers which can diffuse across the cell membrane or by forming channels in the membrane through which ions may pass (Brasseur and Ruysschaert, 1986). One such ionophore, A23187, has been used in a number of different cell systems to increase calcium ion influx. In particular, Dziak et al. (Dziak and Brand, 1974a; Dziak and Brand, 1974b; Dziak and Stern, 1976) have used A23187 to examine the role of calcium in the hormonal regulation of bone cells.

The present study examined the hypothesis that the regulation of alkaline phosphatase and phospholipase A_2 activity by vitamin D_3 metabolites in cartilage cells is mediated by rapid changes in calcium influx. The rapid increase in intracellular Ca^{2+}, postulated to occur in response to 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$, was compared to that of A23187, an agent known to increase intracellular calcium. These experiments examine the effect of ionophore on alkaline phosphatase and phospholipase A_2, two enzymes previously shown to be differentially regulated by these vitamin D metabolites in chondrocyte cultures (Boyan et al., 1988a). Any effects of the ionophore were correlated with the effects of vitamin D metabolites on the cells. Chondrocytes that were preincubated with 1,25-$(OH)_2D_3$ or 24,25-$(OH)_2D_3$ and then exposed to ionophore

were also examined to understand whether there is a synergistic or antagonistic effect of A23187 and hormone.

Material and Methods

Chondrocyte cultures. The culture system used in this study has been described in detail previously (Boyan et al., 1988b) and is outlined below. Rib cages were removed from 125 gram Sprague-Dawley rats by sharp dissection and placed in Dulbecco's modified Eagle's medium (DMEM) until the costochondral cartilages could be removed. The resting zone and adjacent growth zone cartilages were separated, and care was taken to dissect out intervening tissue to limit cross-contamination of cell zones. When the dissection was complete, the cartilages were sliced and then incubated overnight in a 5% CO_2 atmosphere at 37° C. The DMEM was then replaced by two 20-minute washes of Hank's Balanced Salt Solution (HBSS), followed by sequential incubations in 1% trypsin (Gibco, Grand Island, NY) for one hour and 0.02% collagenase (Worthington type II, Freehold, NJ) for three hours. All enzymes were prepared in HBSS. After enzymatic digestion of the extracellular matrix, the cells were separated from tissue debris by filtration through 40-mesh nylon and collected from the filtrate by centrifugation at 500 X g for five minutes, resuspended in DMEM, counted, and plated at an initial density of 10,000 cells/cm^2 for resting zone cells or 25,000 cells/cm^2 for growth zone chondrocytes. Cells were incubated in DMEM containing 10% fetal bovine serum and 50 ug/ml vitamin C in the atmosphere of 5% CO_2

at $37^{\circ}C$ and 100% humidity for 24 hours. The culture media were replaced at that time and then at 72 hour intervals until the cells reached confluence. Cells were subcultured at confluence to T75 flasks at the same plating densities and allowed to return to confluence. Cells were subcultured a maximum of three times to ensure retention of phenotype.

Each vitamin D metabolite was tested at physiological and pharmacological doses. $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ were dissolved in ethanol, since this solvent has been used routinely in other laboratories as the vitamin D vehicle both in vivo and in culture. Before addition to the culture medium, each hormone stock solution was diluted at least 1:5000 (v/v) to minimize any toxic effects of ethanol in the system. The medium that contained the highest concentration of each metabolite was further diluted with DMEM to form media with successively smaller hormone concentrations. Each experiment included control cultures that contained ethanol at the highest concentration used as the hormone vehicle. Both hormones were gifts of Dr. Milan Uskokovic at Hoffman-LaRoche (Nutley, NJ).

A stock solution of the Ca^{2+} ionophore A23187 (Calbiochem, San Diego, CA) was dissolved in dimethylsulfoxide (DMSO) (Fisher Scientific, NJ) at concentrations of 0.01-10 uM. Before addition to the culture medium, each stock solution was diluted at least 1:1000 (v/v) with DMEM to minimize any toxic effects of DMSO in the culture system. Each experiment included control cultures that contained DMSO at the highest concentration used as the ionophore vehicle.

Previous studies (Langston et al., 1989) had shown that the peak in $^{45}Ca^{2+}$ influx in resting zone and growth zone chondrocytes exposed to A23187 occurred within 3 minutes; however, a preliminary experiment demonstrated that the peak in enzyme activity did not occur until 30 minutes after exposure to ionophore. For this reason, enzyme assays were performed on cultures which were incubated with A23187 for 30 minutes. For experiments examining the effects of A23187, the following protocol was used. Fourth passage, confluent cultures of chondrocytes were incubated in DMEM containing vehicle or 0.01 to 10 uM A23187 for 30 minutes prior to harvest. At harvest, the cultures were washed 3 times with DMEM and prepared for enzymatic analysis.

For experiments examining the combined effects of Vitamin D metabolites or A23187, the following protocol was used. Cells were incubated in DMEM containing vehicle or the appropriate vitamin D metabolite for 24 hours prior to harvest. A23187 or vehicle was added to the cultures 30 minutes prior to harvest. In these experiments growth zone chondrocytes were incubated with $1,25-(OH)_2D_3$ and resting zone chondrocytes were incubated with $24,25-(OH)_2D_3$.

Preparation of cell layer. Cell layers were prepared following the method of Hale et al., 1986. Cells were cultured in 24-well culture dishes (Corning, NY). At harvest, the medium was decanted and the cell layer was washed two times with phosphate buffered saline (PBS), then removed using a cell scraper. After centrifugation, the cell layer pellet was washed

again two times with PBS and resuspended by vortexing in 500 ul of deionized water plus 25 ul of 1% Triton X-100. Thus, enzyme assays were performed using the lysates of the cell layers.

Preparation of cell fractions. Matrix vesicles and plasma membranes were prepared as described previously (Boyan et al., 1988b). At harvest, cultures were trypsinized (1% in HBSS), the reaction was stopped with DMEM containing 10% FBS, and the cells were collected by centrifugation at 500 x g for 5 minutes, resuspended in saline, washed twice, and counted. The supernatant from the trypsin digest was centrifuged for 10 minutes at 13,000 X g to pellet a mitochondria/membrane fraction and for 1 hour at 100,000 X g to pellet matrix vesicles.

Plasma membrares were prepared from homogenates of the cell pellets. The chondrocytes were homogenized in a Ten Broek homogenizer, and the plasma membranes were isolated by differential centrifugation, followed by sucrose density centrifugation (Fitzpatrick et al., 1969). Membranes were resuspended in 0.9% NaCl. Detergents such as Triton X-100 were not used to solubilize the membranes, since they inhibit phospholipase A_2. All samples used in subsequent assays represent the combination of three cultures (i.e. three T-75 flasks). The protein content of each fraction was determined (Lowry et al., 1951).

Enzyme assays. Enzyme specific activities were assayed in both the cell layer lysate and in the matrix vesicle and plasma membrane fractions. Alkaline phosphatase [orthophosphoric monoester phosphohydrolase alkaline (EC 3.1.3.1)] was measured

as a function of release of para-nitrophenol from para-nitrophenylphosphate at pH 10.2 (Bretaudiere and Spillman, 1984). Data are expressed in terms of orthophosphate (P_i) produced to facilitate comparisons with previous studies (Schwartz et al., 1988a; Boyan et al., 1988a; Schwartz and Boyan, 1988). There is a one-to-one correlation of uMoles P_i produced with uMoles para-nitrophenol produced.

Phospholipase A_2 (EC 3.1.1.4) was measured as hydrolysis of [^{14}C]arachidonate from [^{14}C]-labeled phosphatidyl-ethanolamine-L-2-R palmitoyl 2, arachidonyl arachidonyl-R^{14}C (New England Nuclear, Boston, MA), as described previously (Schwartz and Boyan, 1988). Data were calculated by comparing free [^{14}C]arachidonate as a percentage of the total ^{14}C in the reaction mixture.

Statistical analysis. Each experiment was performed at least three times. In most instances the data presented below are from typical experiments. Each data point represents the mean $\pm$ SEM (standard error of the mean) for six samples. Treatment/control ratios were derived from 5 independent experiments. Statistical significance was determined by comparing each data point to the control (containing the ethanol vehicle for Vitamin D_3 metabolites and the DMSO vehicle for ionophore solutions) using Student's t test. No differences in response were observed when the control with vehicle was compared to the control without vehicle.

Results

Effect of A23187 on alkaline phosphatase. Alkaline phosphatase specific activity was inhibited by addition of A23187. When alkaline phosphatase in the cell layer was measured (Figure 1), the effect of ionophore was observed at 0.1 and 1 uM in the growth zone chondrocyte cultures but was detectable at only 0.1 uM in the resting zone chondrocyte cultures in the experiment shown. Enzyme specific activity was reduced 2.5 fold in the growth zone chondrocytes. Although the baseline specific activity was considerably lower in the resting zone chondrocyte cultures, specific activity was reduced 6 fold in these cells.

The inhibition of alkaline phosphatase specific activity in cultures incubated with 0.1 uM A23187 was a consistent observation in all replicate experiments. As shown in Figure 2, when the data of 5 independent experiments were analyzed as treatment/control ratios, a significant decrease in enzyme activity was observed at 0.1 uM A23187 in both growth zone and resting zone chondrocyte cultures. In addition, in the experiment used as an example in the results described above, the apparent decrease in alkaline phosphatase activity in resting zone chondrocytes incubated with 1 uM A23187 proved to be statistically significant when the data from multiple experiments were pooled.

The reduction of enzyme activity in response to ionophore was noted in both the matrix vesicles and plasma membranes of both cell types. In resting zone cells (Figure 3), reduction in

Figure 1

THE EFFECT OF IONOPHORE A23187 ON THE SPECIFIC ACTIVITY OF ALKALINE PHOSPHATASE IN THE CELL LAYERS OF CONFLUENT, FOURTH PASSAGE RESTING ZONE (RC) AND GROWTH ZONE (GC) CHONDROCYTES.

Data represent the mean $\pm$ SEM of the cell layers in 6 replicate wells. * Denotes $p < 0.05$ for sample v. control. Data shown are from a single experiment. Each experiment was repeated 5 times.
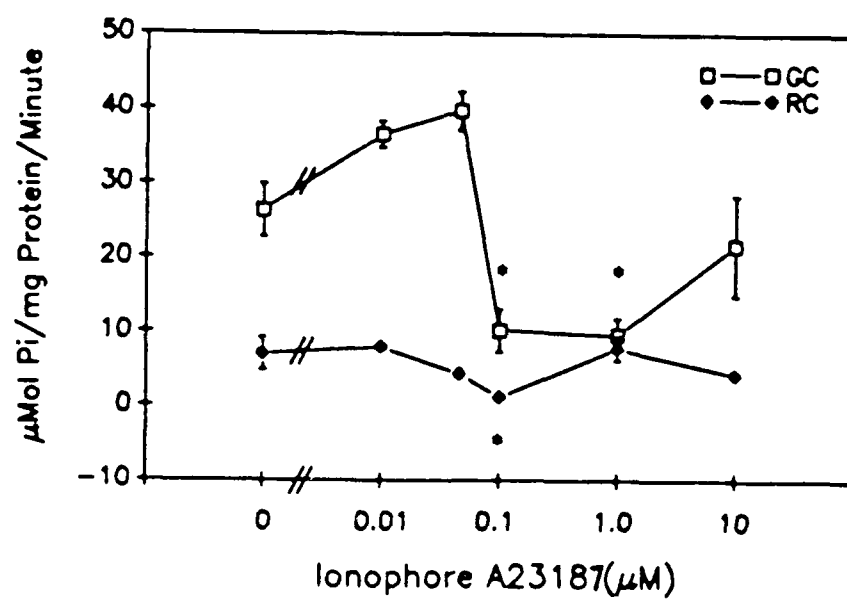
Figure 2

THE EFFECT OF IONOPHORE A23187 ON THE SPECIFIC ACTIVITY OF
ALKALINE PHOSPHATASE IN CONFLUENT, FOURTH PASSAGE CULTURE OF
RESTING ZONE (RC) AND GROWTH ZONE (GC) RAT COSTOCHONDRAL
CHONDROCYTES.

Data from 5 independent experiments are expressed as
treatment/control ratios. Data from each experiment represented
the mean $\pm$ SEM of the cell layers in 6 replicate walls. *
Denotes $p < 0.05$ for sample v. control.

Figure 3

THE EFFECT OF IONOPHORE A23187 ON THE SPECIFIC ACTIVITY OF
ALKALINE PHOSPHATASE IN PLASMA MEMBRANES AND MATRIX VESICLES
ISOLATED FROM CONFLUENT, FOURTH PASSAGE RESTING ZONE
CHONDROCYTES.

Data represent the mean $\pm$ SEM of 6 samples. Each sample
represents the combined membranes from 3 T-75 flasks.

* Denotes $p < 0.05$ for sample v. control. Data shown are
from a single experiment. Each experiment was repeated 3 times.

y-axis: μMol Pi/mg Protein/Minute

Legend:
□ MV
● PM

x-axis: Ionophore A23187 (μM)
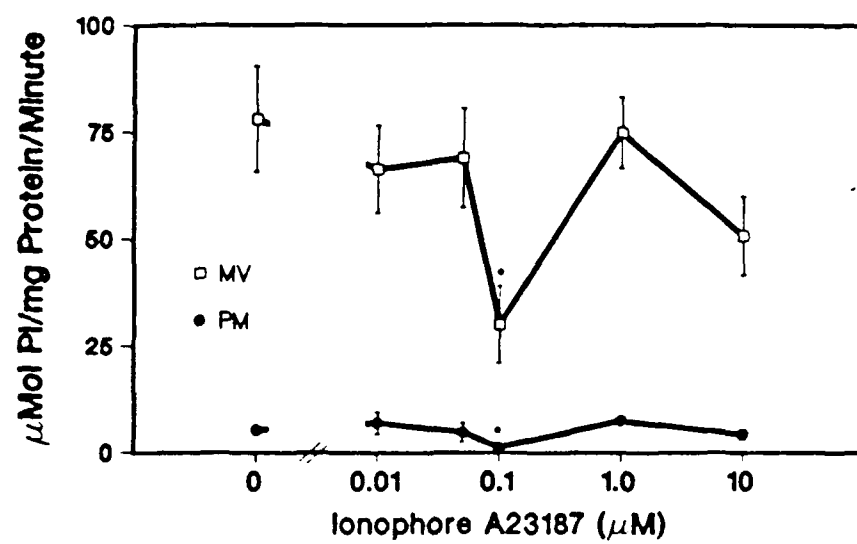
enzyme specific activity was noted only at 0.1 uM A23187 in both the matrix vesicles and plasma membranes. Specific activity in the matrix vesicles was reduced to 31% of the basal activity. Plasma membranes exhibited a comparable percent reduction in specific activity. However, since basal activity in the matrix vesicles was 15 fold greater than in the plasma membranes, the absolute magnitude of reduction was also greater.

Inhibition of alkaline phosphatase specific activity was also observed in matrix vesicles and plasma membranes isolated from growth zone chondrocytes in response to ionophore (Figure 4). In both instances the effect of 0.1 uM A23187 was to reduce activity to 57% that of the untreated control. Enzyme activity was further decreased at 1 uM A23187 to 38% of the untreated control in the matrix vesicles only. While alkaline phosphatase specific activity was 3 fold greater in the matrix vesicles isolated from untreated cultures when compared to the plasma membranes, the effect of ionophore was to decrease the effective fold enrichment of enzyme activity to 1.8 at 0.1 uM and to 1 at 1 uM A23187.

When data are calculated as a function of cell number (Table I), the effect of ionophore is evident at 0.1 and 1 uM A23187 in matrix vesicles isolated from growth zone and resting zone chondrocyte cultures. The reduction in enzyme activity at 0.1 uM is 2.3 fold for matrix vesicles isolated from growth zone chondrocyte cultures and 2.9 fold for matrix vesicles isolated from resting zone cultures. At 1 uM A23187, the effect of ionophore is less, producing a 1.4-1.5 fold decrease compared to

Figure 4

THE EFFECT OF IONOPHORE A23187 ON THE SPECIFIC ACTIVITY OF ALKALINE PHOSPHATASE IN CONFLUENT, FOURTH PASSAGE RAT COSTOCHONDRAL GROWTH ZONE CHONDROCYTES.

Data represent the mean ± SEM of 6 samples. Each sample represents the combined plasma membranes or matrix vesicles from 3 T-75 flasks. * Denotes $p < 0.05$ for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.

Ionophore A23187 (μM)
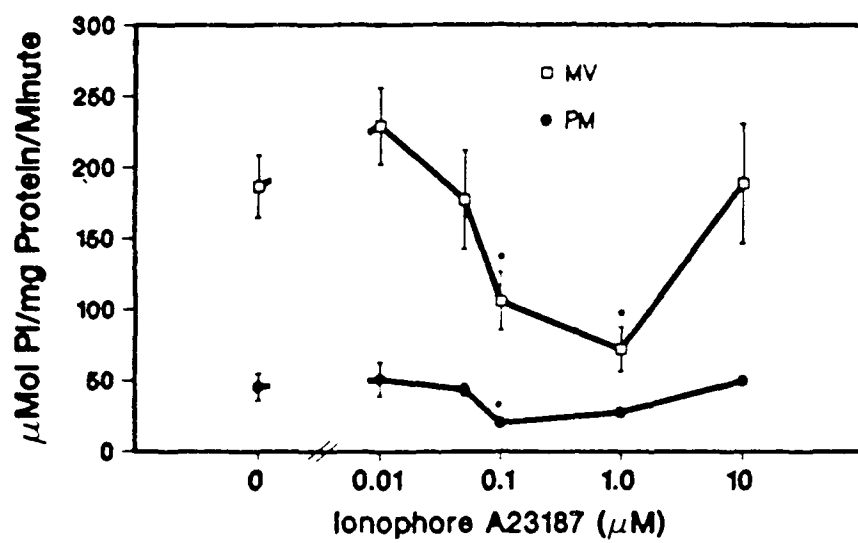
Table I: The effect of A23187 on the activity of alkaline phosphatase in confluent, fourth passage cultures of rat costochondral growth zone (GC) and resting zone (RC) chondrocytes.

[uM A23187]	uMol $P_i/10^6$ Cells/Minute			
	Matrix Vesicles		Plasma Membranes	
	GC	RC	GC	RC
0	1.3 ± 0.2	0.6 ± 0.1	4.1 ± 0.5	0.9 ± 0.2
0.01	1.5 ± 0.3	0.6 ± 0.1	3.4 ± 0.9	1.5 ± 0.5
0.05	1.6 ± 0.4	0.4 ± 0.1	3.2 ± 0.5	0.5 ± 0.1
0.10	0.6 ± 0.2*	0.2 ± 0.0*	2.5 ± 0.3*	0.3 ± 0.0*
1.00	0.9 ± 0.2*	0.4 ± 0.1	1.8 ± 0.2*	1.3 ± 0.4
10.00	1.6 ± 0.3	0.4 ± 0.2	5.6 ± 1.5	0.4 ± 0.0

Each point is the mean ± SEM of 6 samples, each sample being derived from the matrix vesicles or plasma membranes isolated from 3 T-75 flasks. *Denotes $p < 0.05$ for treatment v. control. Data shown are from a single experiment and each experiment was repeated 3 times.

control matrix vesicles. Statistically significant decreases in enzyme activity were observed at 0.1 uM in the plasma membranes of both cell types. Maximal inhibition was observed at 1 uM A23187 in the membranes isolated from the growth cartilage cells only.

Phospholipase A_2. Although there was an apparent reduction in phospholipase A_2 activity in matrix vesicles and plasma membranes of both growth zone and resting zone chondrocytes in response to 0.1 uM A23187, incubation of the chondrocytes with A23187 had no statistically significant effect on phospholipase A_2 specific activity (Table II).

Effect of A23187 on vitamin D-stimulated alkaline phosphatase activity. When growth zone chondrocytes (Figure 5) were preincubated with 10^{-8} M $1,25-(OH)_2D_3$, there was a stimulation of enzyme activity in the matrix vesicles but not in the plasma membranes. Cultures which were incubated with 0.1 uM A23187 exhibited a reduction in enzyme activity in both the plasma membranes and matrix vesicles. When cells that had been preincubated with 10^{-8} M $1,25-(OH)_2D_3$ were challenged with 0.1 uM A23187, there was an inhibition of the hormone-stimulated alkaline phosphatase to levels achieved by ionophore alone. When the hormone concentration in the pre-incubation medium was reduced to 10^{-9} M $1,25-(OH)_2D_3$, challenge with 0.1 uM A23187 resulted in statistically significant reductions in alkaline phosphatase activity in matrix vesicles but not in the plasma membranes.

Table II: The effect of ionophore A23187 on the phospholipase A_2 activity in matrix vesicles and plasma membranes isolated from confluent, fourth passage cultures of growth zone (GC) and resting zone (RC) rat costochondral chondrocytes.

[uM A23187]	Percent Hydrolysis/mg Protein/Minute			
	Matrix Vesicles		Plasma Membranes	
	GC	RC	GC	RC
0	5.7 ± 1.9	10.2 ± 1.3	3.6 ± 0.7	4.4 ± 1.2
0.01	2.9 ± 0.9	7.9 ± 1.0	3.3 ± 0.7	2.9 ± 0.6
0.05	2.1 ± 0.4	10.6 ± 1.3	3.3 ± 0.7	4.8 ± 1.9
0.10	2.1 ± 0.3	7.3 ± 1.6	2.2 ± 0.3	2.9 ± 0.4
1.00	2.4 ± 0.^{3}	11.5 ± 2.6	2.3 ± 0.8	3.3 ± 1.3
10.00	4.8 ± 0.3	11.9 ± 1.9	3.0 ± 1.1	7.9 ± 2.8

Each point is the mean ± SEM of 6 samples, each sample being derived from the matrix vesicles or plasma membranes isolated from 3 T-75 flasks. Data shown are from a single experiment and each experiment was repeated 3 times.

Figure 5.

THE EFFECT OF IONOPHORE A23187 AND $1,25-(OH)_2D_3$ ON THE SPECIFIC ACTIVITY OF ALKALINE PHOSPHATASE IN CONFLUENT, FOURTH PASSAGE CULTURES OF RAT COSTOCHONDRAL GROWTH ZONE CHONDROCYTES.

Matrix vesicles and plasma membranes isolated from 3 T-75 flasks were combined for each sample. Data represent the mean $\pm$ SEM of 6 samples. * Denotes $p < 0.05$ for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.

μMol Pi/mg Protein/Minute

MV
PM

50
40
30
20
10
0

1,25-(OH)$_2$D$_3$(M)   0   0 10^{-8} 10^{-8} 10^{-8} 10^{-9}
A23187 (μM)   0   0.1   0   1   0.1   10
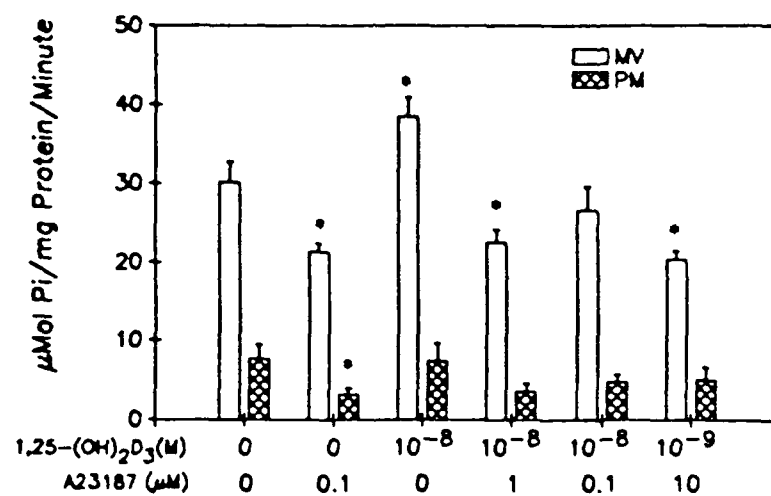
Resting zone cell cultures (Figure 6) exhibited similar phenomena in response to 24,25-(OH)$_2$D$_3$. Cultures incubated with 0.1 uM A23187 alone demonstrated a statistically significant decrease in alkaline phosphatase specific activity in both the plasma membranes and matrix vesicles. When these cells were pre-incubated with 10^{-7} M 24,25-(OH)$_2$D$_3$, there was a significant increase in enzyme activity in the matrix vesicles only. However, when 1 uM A23187 was added to the cultures preincubated with 10^{-7} M 24,25-(OH)$_2$D$_3$, alkaline phosphatase activity was reduced in both membrane fractions to levels comparable to cells incubated with ionophore alone.

The effect of ionophore was dose dependent. When 24,25-(OH)$_2$D$_3$ concentration was held constant at 10^{-7} M, addition of 0.1 uM A23187 resulted in a reduction in enzyme activity in both membrane fractions but the reduction was significant in the plasma membranes only. However, at 0.01 uM A23187, ionophore had no effect on the 24,25-(OH)$_2$D$_3$-dependent stimulation. In cultures preincubated with 10^{-8} or 10^{-9} M 24,25-(OH)$_2$D$_3$, addition of 0.1 uM A23187 reduced alkaline phosphatase to levels comparable to cells incubated with A23187 alone.

Discussion

Independent studies in our laboratory have shown that incubation with A23187 results in a rapid increase in the rate of ^{45}Ca^{2+} uptake by both growth zone and resting zone chondrocytes (Langston et al., 1989). This effect of the ionophore has also been demonstrated in other systems such as

Figure 6

THE EFFECT OF IONOPHORE A23187 AND $24,25-(OH)_2D_3$ ON THE SPECIFIC ACTIVITY OF ALKALINE PHOSPHATASE IN CONFLUENT, FOURTH PASSAGE CULTURES OF RAT COSTOCHONDRAL RESTING ZONE CHONDROCYTES.

Matrix vesicles and plasma membranes isolated from 3 T-75 flasks were combined for each sample. Data represent the mean ± SEM of 6 samples. * Denotes $p < 0.05$ for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.

The figure's y-axis is labeled "μMol Pi/mg Protein/Minute" with values 0, 5, 10, 15, 20, 25. Legend: MV, PM.

24,25-(OH)$_2$D$_3$(M) 0   0 10^{-7} 10^{-7} 10^{-7} 10^{-7} 10^{-8} 10^{-9}

A23187 (μM) 0   0.1   0   1   0.1   0.01   0.1   0.1
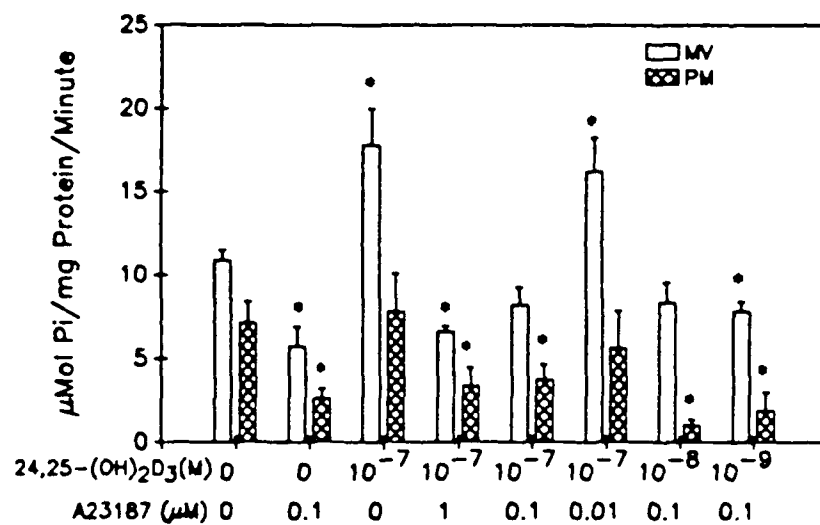
fetal rat bone (Dziak and Stern, 1976). In both bone cells and chondrocytes, $^{45}Ca^{2+}$ uptake was rapid, occurring within 3 minutes of exposure to ionophore. While optimum $^{45}Ca^{2+}$ influx reported by Dziak and Stern (1976) occurred at 10^{-5} M A23187, the highest level of $^{45}Ca^{2+}$ uptake in the chondrocyte cultures was at 10^{-7} M (0.1 uM) A23187, suggesting that there may be differences in the interaction of the ionophore with osteoblast and chondrocyte membranes.

The concentration of A23187 at which maximum ion uptake occurred (Langston et al., 1989) also coincided with the optimum effect of ionophore on all other parameters studied. This supported the concept that the effects measured in the present study were related to $^{45}Ca^{2+}$ influx and not to the physical presence of the ionophore per se. It is unlikely that the effects observed are due to ion efflux in the chondrocytes because Dziak and Stern (1976) had established that A23187 had no effect on $^{45}Ca^{2+}$ efflux in bone cells. It is possible that A23187 induced the redistribution of $^{45}Ca^{2+}$ within the cell in addition to, or rather than, promoting influx of extracellular $^{45}Ca^{2+}$. This does not necessarily change the interpretation of the data, however, since the intent of the experiments was to compare the effects of an agent known to alter Ca^{2+} flux with the effects of hormones hypothesized to alter Ca^{2+} flux.

Our previous studies (Langston et al., 1989) indicated that the effects of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ on $^{45}Ca^{2+}$ flux are distinctly different and dose-dependent. In addition, neither metabolite influenced $^{45}Ca^{2+}$ flux in a manner comparable to that

of A23187. Thus, any effects of A23187 on enzyme activity due to $^{45}Ca^{2+}$ do not reflect the changes in chondrocytes induced by either of the vitamin D metabolites. It is possible that vitamin D-induced changes in intracellular calcium are important in the mechanism of hormone action in these cells. For example, if 1,25-(OH)$_2$D$_3$-stimulated Ca^{2+} flux were to result in a reduction of intracellular Ca^{2+} concentration, the opposite effects of 1,25-(OH)$_2$D$_3$ and A23187 on alkaline phosphatase might be predicted.

It is certainly possible that at least some of the changes observed in response to ionophore were due to effects on the cell membranes that were independent of ion movements. It is unlikely that this was a general effect since incubation with the ionophore had no statistically significant effect on phospholipase A$_2$ activity although this enzyme, like alkaline phosphatase, is associated with plasma membranes. Some reduction in enzyme activity may have been due to differences in susceptibility to trypsin digestion in cells incubated with ionophore as compared to controls or vitamin D-treated cultures. If this were the case, one would expect both enzymes to have been affected and for the reduction in alkaline phosphatase observed at 0.1 uM A23187 to have been observed over a larger dose range of ionophore.

Recent investigators have reported the multiplicity of actions of 1,25-(OH)$_2$D$_3$, including a rapid direct action in altering intestinal membrane transport of Ca^{2+} prior to the synthesis of specific transport proteins (Nemere and Norman,

1986; Wassermman and Fullmer, 1983; Fullmer et al., 1984; Yoshimoto and Norman, 1986; Wasserman and Taylor, 1969). This rapid effect has been observed in bone forming cells as well. In mouse osteoblasts, $1,25-(OH)_2D_3$ induces immediate transient increases in cytosolic Ca^{2+} (Lieberherr, 1987). Matsumoto et al. (1981) have demonstrated that the synthesis of phosphatidylcholine and incorporation of arachidonic acid in response to $1,25-(OH)_2D_3$ correlates with increased cytosolic calcium, and have hypothesized that this calcium functions as a "second messenger," regulating numerous cellular pathways and events.

Previous data from our laboratory (Schwartz et al., 1988a) have demonstrated that chondrocyte membrane enzymes can be regulated by $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ in the absence of any gene transcription or translation through a direct interaction with the membrane, suggesting that transmembrane ion fluxes may play a role. The present observations support the concept that intracellular calcium concentration can be a regulatory influence on alkaline phosphatase activity (Feher and Wasserman, ₋979; Schiffl and Binswanger, 1980), since this enzyme was inhibited at concentrations of A23187 that also resulted in increased $^{45}Ca^{2+}$ in the cells.

It is unlikely, however, that the effects of vitamin D metabolites on the cells is due to the rapid increase in cell $^{45}Ca^{2+}$ content like that produced by the ionophore. In our culture system, A23187, in concentrations that caused increased $^{45}Ca^{2+}$ influx, caused an inhibition of alkaline phosphatase

specific activity. However, earlier reports have shown that the vitamin D metabolites stimulate alkaline phosphatase specific activity in this chondrocyte model (Boyan et al., 1988a).

A23187 inhibited enzyme activity in both the plasma membrane and matrix vesicles whereas the effect of the vitamin D metabolites on the chondrocytes in culture is on the matrix vesicles only. This suggests that any effects on enzyme activity by hormone-induced Ca^{2+} movements must be regulated differently than those induced by A23187-dependent Ca^{2+} influx. This hypothesis is further supported by the observation that enzyme activity is stimulated by vitamin D metabolites in isolated matrix vesicles and plasma membranes incubated directly with hormone (Schwartz et al., 1988a), just as the effects of ionophore may override cellular regulation of the plasma membrane enzyme.

Previous studies have also shown that regulation of membrane enzymes by vitamin D metabolites is metabolite-specific and dependent upon the state of chondrocyte differentiation (Boyan et al., 1988a; Schwartz and Boyan, 1988; Schwartz et al., 1988b). Alkaline phosphatase activity in the growth zone chondrocyte cultures is stimulated by $1,25-(OH)_2D_3$ whereas it is stimulated by $24,25-(OH)_2D_3$ in the resting zone cell cultures. However, the effects of ionophore were independent of cell type, indicating that simply elevating calcium ion content of the cell or matrix vesicle cannot account for the regulation of enzymatic activity in response to the vitamin D metabolites.

Furthermore, phospholipase A_2 activity is regulated by the vitamin D metabolites in a differential manner. Phospholipase A_2 is stimulated in matrix vesicles produced by growth zone chondrocytes incubated with $1,25-(OH)_2D_3$ whereas it is inhibited by $24,25-(OH)_2D_3$ in matrix vesicles produced by resting zone chondrocytes (Schwartz and Boyan, 1988). The differential effect of hormone on this enzyme is seen in isolated matrix vesicles incubated directly with the metabolites as well (Schwartz et al., 1988a). Although phospholipase A_2 is known to be regulated by calcium (de Haas et al., 1971; Wells, 1972), no statistically significant effect was observed in the present study as a function of A23187. This suggests that, as with alkaline phosphatase, Ca^{2+} content alone is not sufficient to account for the effects of hormone on this enzyme.

The effects of vitamin D metabolites and A23187 appear to be antagonistic. Although incubation with the appropriate metabolite stimulates alkaline phosphatase activity in the matrix vesicles at both stages of chondrocyte differentiation, this stimulation can be inhibited by addition of ionophore, and by inference, Ca^{2+}. A23187 also inhibits the plasma membrane enzyme in these cultures, which has been shown previously not to respond to hormone under these experimental conditions (Schwartz and Boyan, 1988). Inhibition of $1,25-(OH)_2D_3$-stimulated alkaline phosphatase in growth zone cells or $24,25-(OH)_2D_3$-stimulated enzyme in resting zone cells requires an order of magnitude more ionophore than is necessary in unstimulated cells. As hormone concentration is decreased, less ionophore is

necessary to block stimulation, however, even at these lower hormone concentrations, A23187-dependent decreases in alkaline phosphatase are reduced.

These observations suggest that the rapid increase in alkaline phosphatase activity observed in response to vitamin D metabolites is independent of any rapid transient increases in Ca^{2+} that might be associated with hormone action on the cell membrane. Recent reports (Levy et al., 1987) indicating that $1,25-(OH)_2D_3$ alters membrane phospholipid composition and enhances calcium efflux in HL-60 cells, suggest that the hormones may be acting in a similar fashion in the chondrocyte cultures as well. This would explain, at least in part, the antagonistic effects of the vitamin D metabolites and A23187 described. Preliminary observation in our laboratory support this hypothesis.

The data presented in this study corroborate those previously reported by this laboratory (Schwartz et al., 1988b) that subtle regulation of chondrocyte alkaline phosphatase may be missed if one only examines the cell layer and not the individual membrane fractions. The sensitivity afforded by measuring effects on matrix vesicles is significant. For instance, alkaline phosphatase activity in the growth zone chondrocyte cell layer is 260 uMol P_i/mg protein/minute. Specific activity in the plasma membrane is 190 uMol P_i/mg protein/minute, but in the matrix vesicles it is 750 uMol P_i/mg protein/minute. Similarly, specific activity in the resting zone chondrocyte cell layer is 70, in the plasma membranes it is

6, and in the matrix vesicles, it is approximately 80 uMol P_i/mg protein/minute. Although the percent inhibition of alkaline phosphatase in response to ionophore is the same for both membrane populations, the effect of ionophore, like that of the vitamin D metabolites, is greatest in the matrix vesicle fraction. Thus, any changes in enzyme activity must, as a matter of course, have their predominant effect on the matrix vesicle, and, as a consequence, on its function in the extracellular matrix.

Antagonistic Effects of A23187 on $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ Dependent Stimulation of Alkaline Phosphatase Activity in Costochondral Chondrocyte Cultures. Schwartz, Z., Langston, G.G., Swain, L.D., Boyan, B.D. Endocrinology, submitted for publication, 1989.

Chapter II

EFFECT OF 1,25-(OH)$_2$D$_3$ AND 24,25-(OH)$_2$D$_3$ ON CALCIUM

ION FLUXES IN COSTOCHONDRAL CHONDROCYTE CULTURES

44

Summary

Vitamin D_3 metabolites have been shown to affect proliferation, differentiation and maturation of cartilage cells. Previous studies have shown that growth zone chondrocytes respond primarily to $1,25-(OH)_2D_3$ whereas resting zone chondrocytes respond primarily to $24,25-(OH)_2D_3$. To examine the role of calcium in the mechanism of hormone action, this study examined the effects of the Ca^{2+} ionophore A23187, $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ on $^{45}Ca^{2+}$ influx and efflux in growth zone chondrocytes and resting zone chondrocytes derived from the costochondral junction of 125g rats. Influx was measured as incorporation of $^{45}Ca^{2+}$. Efflux was measured as release of $^{45}Ca^{2+}$ from pre-labelled cultures into fresh media. The pattern of $^{45}Ca^{2+}$ influx in unstimulated (control) cells over the incubation period was different in the two chondrocyte populations, whereas the pattern of efflux was comparable. A23187 induced a rapid influx of $^{45}Ca^{2+}$ in both types of chondrocytes which peaked by 3 minutes and was over by 6 minutes. Influx was greatest in the growth zone chondrocytes. Addition of 10^{-8} to 10^{-9} M $1,25-(OH)_2D_3$ to growth zone chondrocyte cultures resulted in a dose dependent increase in $^{45}Ca^{2+}$ influx after 15 minutes. Efflux was stimulated by these concentrations of hormone throughout the incubation period. Addition of 10^{-6} to 10^{-7} M $24,25-(OH)_2D_3$ to resting zone chondrocytes resulted in an inhibition in ion efflux between 1-6 minutes, with no effect on influx during this period. Efflux returned to control values between 6-15 minutes. $^{45}Ca^{2+}$ influx

was inhibited by these concentrations of hormone from 15-30 minutes. These studies demonstrate that changes in Ca^{2+} influx and efflux are metabolite-specific and may be a mechanism by which vitamin D metabolites directly regulate chondrocytes in culture.

Introduction

Costochondral cartilage chondrocyte cultures have been used as a model to examine the characteristics of resting zone chondrocytes and growth zone chondrocytes in order to understand the process of their differentiation and regulation during endochondral ossification (Boyan et al., 1988a). These studies have shown that the response of the chondrocytes to $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ in vitro can be used as markers of their differentiation (Boyan et al., 1988b). For example, alkaline phosphatase and phospholipase A_2 specific activities are elevated when growth zone chondrocytes are incubated with $1,25-(OH)_2D_3$ but this metabolite has no effect on either enzyme in resting zone chondrocyte cultures. In contrast, the $24,25-(OH)_2D_3$ metabolite increases alkaline phosphatase activity but inhibits phospholipase A_2 in resting zone cells and has no effect on either enzyme in growth zone chondrocyte cultures (Schwartz and Boyan, 1988). These observations demonstrate that the actions of these hormones are cell and metabolite-specific.

Many of the effects of vitamin D_3 on chondrocytes may be due to changes in intracellular Ca^{2+} concentration (Tanaka and DeLuca, 1971). $1,25-(OH)_2D_3$ stimulates calcium transport in the

intestine without <u>de</u> <u>novo</u> protein synthesis (Tanaka and DeLuca, 1971; Bikle et al., 1978). Data from our laboratory (Schwartz and Boyan, 1988), using isolated matrix vesicles produced by chondrocytes in culture, indicates that $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ can alter the specific activities of alkaline phosphatase and phospholipase A_2, which is modulated by calcium (Wells, 1972) via direct interaction with the membrane under conditions where no protein synthesis is possible. It is postulated that vitamin D_3 metabolites initiate changes in the phospholipid composition of cell membranes with resultant modifications in fluidity and permeability (Boyan et al., 1988a; Rasmussen et al., 1982; Putkey et al., 1986). Thus, calcium transport could occur by changing the physical characteristics of the membrane lipid components (Levy et al., 1987).

Recently, we have shown that incubation with the ionophore A23187 can inhibit the $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ dependent stimulation of alkaline phosphatase in chondrocyte cultures, suggesting that rapid influx of Ca^{2+} may be antagonistic to hormone action (unpublished observations). It is possible that calcium ion fluxes in response to ionophore are not comparable to those produced by $1,25-(OH)_2D_3$ or $24,25-(OH)_2D_3$. In the present study, we examined the dose dependence and time course of response to these two vitamin D metabolites in chondrocytes shown previously to be their primary target cell with respect to enzyme activity (Boyan et al., 1988b; Schwartz and Boyan, 1988), to assess whether Ca^{2+} flux is differentially regulated. Vitamin D-dependent $^{45}Ca^{2+}$ flux was compared to that of the

ionophore A23187 which enhances Ca^{2+} transport across lipid membranes (Brasseur and Ruysschaert, 1986).

Material and Methods

Chondrocyte cultures. The culture system used in this study has been described in detail previously (Boyan et al., 1988a). Rib cages were removed from 125g Sprague-Dawley rats, placed in Dulbecco's modified Eagle's medium (DMEM) and the costochondral cartilages removed. The resting zone and adjacent growth zone cartilages were separated, and intervening tissue removed to limit cross-contamination of cell zones. Following dissection, the cartilages were sliced and then incubated overnight in 5% CO_2 atmosphere at 37°C. The DMEM was then replaced by two 20-minute washes of Hank's Balanced Salt Solution (HBSS), followed by sequential incubations in 1% trypsin (Gibco, Grand Island, NY) for one hour and 0.02% collagenase (Worthington type II, Freehold, NJ) for three hours. All enzymes were prepared in HBSS. After enzymatic digestion of the extracellular matrix, the cells were separated from tissue debris by filtration, collected by centrifugation, resuspended in DMEM, counted, and plated at an initial density of 10,000 cells/cm^2 for resting zone cells or 25,000 cells/cm^2 for growth cartilage chondrocytes. Cells were incubated in DMEM containing 10% fetal bovine serum and 50 ug/ml vitamin C in the atmosphere of 5% CO_2 at 37°C and 100% humidity for 24 hours. The culture media were replaced at that time and then at 72 hour intervals. Cells were subcultured at confluence to T75 flasks at the same

plating densities and allowed to return to confluence. Cells were subcultured a maximum of four passages to ensure retention of phenotype.

Each vitamin D metabolite was tested at physiological and pharmacological doses. $1,25\text{-}(OH)_2D_3$ and $24,25\text{-}(OH)_2D_3$ were dissolved in ethanol, routinely used in other laboratories as a vitamin D vehicle. Before addition to the culture medium, each hormone stock solution was diluted at least 1:5000 (v/v) to minimize any toxic effects of ethanol. Each metabolite was further diluted with DMEM to form media with successively smaller hormone concentrations and each experiment included control cultures that contained ethanol at the highest concentration used as the hormone vehicle. Both hormones were gifts of Dr. Milan Uskokovic at Hoffman-LaRoche (Nutley, NJ).

A stock solution of the Ca^{2+} ionophore A23187 (Calbiochem, San Diego, CA) was dissolved in dimethylsulfoxide (DMSO) at concentrations of 0.01-10 uM. Before addition to the culture medium, each stock solution was diluted at least 1:1000 (v/v) with DMEM to minimize any toxic effects of DMSO in the culture system. Each experiment included control cultures that contained DMSO at the highest concentration used as the ionophore vehicle.

Preparation of cell layer. Cell layers were prepared following the method of Hale et al. (1986). Cells were cultured in 24-well culture dishes (Corning, NY). At harvest, the medium was decanted and the cell layer was washed two times with phosphate buffered saline (PBS), then removed using a cell

scraper. After centrifugation, the cell layer pellet was washed with PBS and resuspended by vortexing in 500 ul of deionized water plus 25 ul of 1% Triton X-100.

Ca^{2+} **influx.** Calcium influx was measured as the uptake of $^{45}Ca^{2+}$ by confluent, fourth passage chondrocytes utilizing a modification of the method of Dziak and Brand (1974a, 1974b). Radiolabelled calcium (10 uCi/ml $^{45}Ca^{2+}$, New England Nuclear) was added to the cells with vitamin D metabolites or ionophore A23187 to minimize any artifact that might be due to shifts in intracellular $^{45}Ca^{2+}$ pools in prelabelled cultures. At harvest the cells were washed three times with PBS and the cell layer then removed using a cell scraper and the radioactivity in the cell layer determined by liquid scintillation spectroscopy.

Ca^{2+} **efflux.** Calcium efflux was measured as the release of $^{45}Ca^{2+}$ from confluent, fourth passage chondrocytes preloaded with isotope via incubation for 20 hours in media containing 10 uCi/ml $^{45}Ca^{2+}$. At the end of the loading period the cells were washed three times with DMEM and then DMEM containing vitamin D metabolites was added to the cells. Efflux was measured by taking 50 ul aliquots of the media at 1,3,6,9,15,30, and 60 minutes for growth zone chondrocytes and at 1,6,15,30 and 60 minutes for resting zone cells. Radioactivity was determined by liquid scintillation spectroscopy.

Statistical analysis. Each experiment was performed at least three times. Data presented are from typical experiments. Each data point is the mean $\pm$ SEM for six samples. Statistical significance was determined by comparing each data point to the

control (containing the ethanol vehicle for Vitamin D_3 metabolites and the DMSO vehicle for ionophore solutions) using Student's t test. No differences in response were observed when the control with vehicle was compared to the control without vehicle.

Results

Calcium Influx.

A23187. A23187 at 0.1 and 1 uM stimulated $^{45}Ca^{2+}$ uptake in resting zone chondrocytes (Figure 7). The effect of ionophore was rapid; it was evident within 1 minute and peaked in 3 minutes. At longer time intervals, experimental groups did not significantly differ from controls. Growth zone cells (Figure 8) also showed a significant increase in $^{45}Ca^{2+}$ influx at 1 and 3 minutes with 0.1 and 1 uM A23187. However, the degree of stimulation was much greater in the growth zone chondrocytes than resting zone chondrocytes.

In addition, the overall pattern of $^{45}Ca^{2+}$ uptake was distinctly different in the two cell types. Resting zone cells continued to import $^{45}Ca^{2+}$ over the first 15 minutes of the 30 minute incubation period, whether or not A23187 was present (Figure 7). By 6 minutes, there was no longer any additive effect of ionophore. In growth zone chondrocytes, $^{45}Ca^{2+}$ uptake was decreased to unstimulated control values by 6 minutes and remained there for the remainder of the 30 minute incubation whether or not A23187 was present (Figure 8).

Figure 7

THE EFFECT OF IONOPHORE A23187 ON $^{45}Ca^{2+}$ INFLUX IN CONFLUENT, FOURTH PASSAGE RESTING ZONE CHONDROCYTES FROM 1-30 MINUTES.

Data represent the mean $\pm$ SEM of 6 samples. * Denotes $p <$ 0.05 for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.
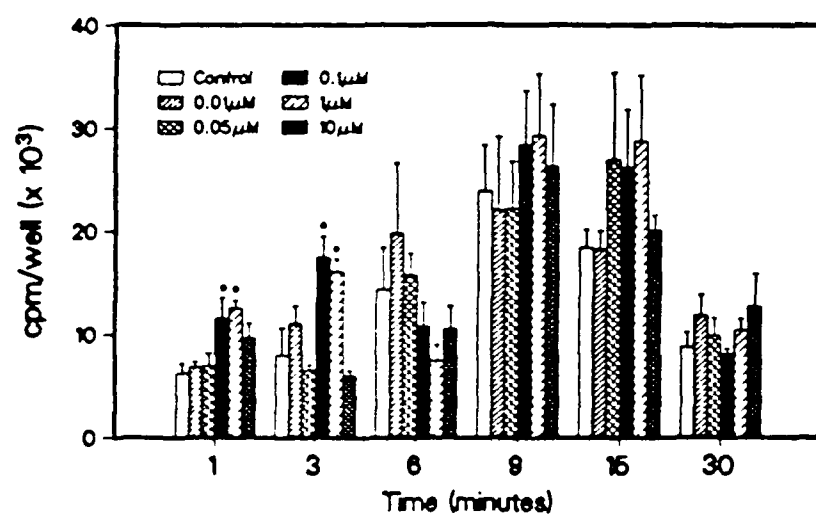
Legend:
□ Control   ■ 0.1μM
▨ 0.01μM   ▨ 1μM
▨ 0.05μM   ■ 10μM

y-axis: cpm/well (x 10³)
x-axis: Time (minutes)

Figure 8

THE EFFECT OF IONOPHORE A23187 ON $^{45}Ca^{2+}$ INFLUX IN CONFLUENT, FOURTH PASSAGE GROWTH ZONE CHONDROCYTES FROM 1-3 MINUTES.

Data represent the mean $\pm$ SEM of 6 samples. * Denotes $p <$ 0.05 for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.
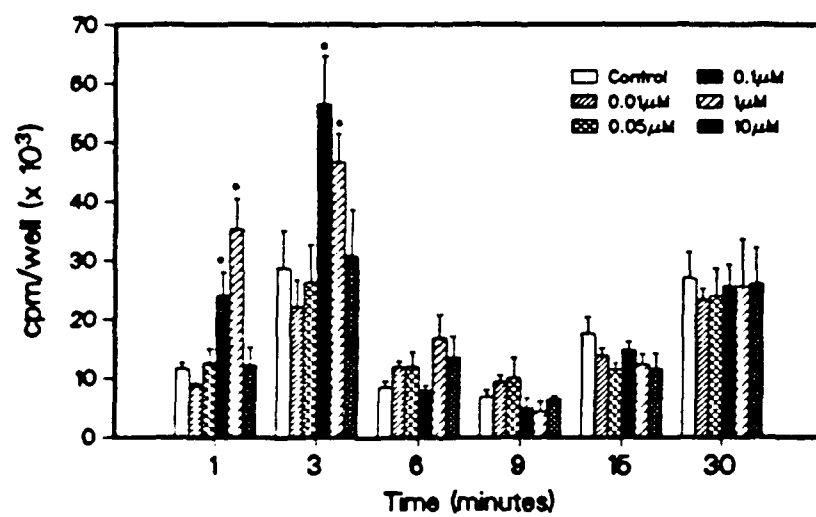
Figure 9 illustrates the difference in magnitude of response between growth zone cells and resting zone cells at a single point (3 minutes) and increasing ionophore concentration. The elevation in $^{45}Ca^{2+}$ content was statistically significant at both 0.1 and 1.0 uM ionophore in both cell types. $^{45}Ca^{2+}$ influx was 3.7 fold greater in unstimulated growth zone chondrocytes. Although the absolute increase in calcium uptake was greater in the growth zone cells, the rate of increase was essentially the same for both (growth zone cells: 2.1 X; resting zone cells: 2.2 X). As a result, at 0.1 uM A23187, the magnitude of difference between the two cells remained the same.

Vitamin D metabolites. When resting zone cells were incubated with $24,25-(OH)_2D_3$ (Figure 10), the effect on $^{45}Ca^{2+}$ influx was markedly different from that induced by A23187. $^{45}Ca^{2+}$ influx continued to increase over the 30 minute incubation period in the control cells and in cells incubated with low doses of hormone (10^{-8} to 10^{-9} M). In cultures incubated with 10^{-6} to 10^{-7} M $24,25-(OH)_2D_3$, $^{45}Ca^{2+}$ uptake was reduced to initial values at 15 minutes and remained reduced through the 30 minute test period. Compared to control, at 15 minutes $24,25-(OH)_2D_3$ reduced $^{45}Ca^{2+}$ uptake 2.4 fold.

The change in $^{45}Ca^{2+}$ influx induced by $1,25-(OH)_2D_3$ in growth zone cells (Figure 11) was opposite to that seen in resting zone chondrocytes stimulated with $24,25-(OH)_2D_3$. Influx was significantly elevated at 15 and 30 minutes by 10^{-9} and 10^{-8} M hormone. At 15 minutes, $^{45}Ca^{2+}$ influx was increased 2.8 fold

Figure 9

THE EFFECT OF A23187 ON $^{45}Ca^{2+}$ INFLUX IN CONFLUENT FOURTH PASSAGE RESTING ZONE CHONDROCYTES (RC) AND GROWTH ZONE CHONDROCYTES (GC) AFTER 3 MINUTES OF EXPOSURE TO IONOPHORE.

Data represent the mean ± SEM of 6 samples. * Denotes $p <$ 0.05 for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.

Ionophore A23187 (μM)

Figure 10

THE EFFECT OF 24,25-(OH)$_2$D$_3$ ON ^{45}Ca^{2+} INFLUX IN CONFLUENT, FOURTH PASSAGE RESTING ZONE CHONDROCYTES FROM 1-30 MINUTES.

Data represent the mean ± SEM of 6 samples. * Denotes p < 0.05 for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.
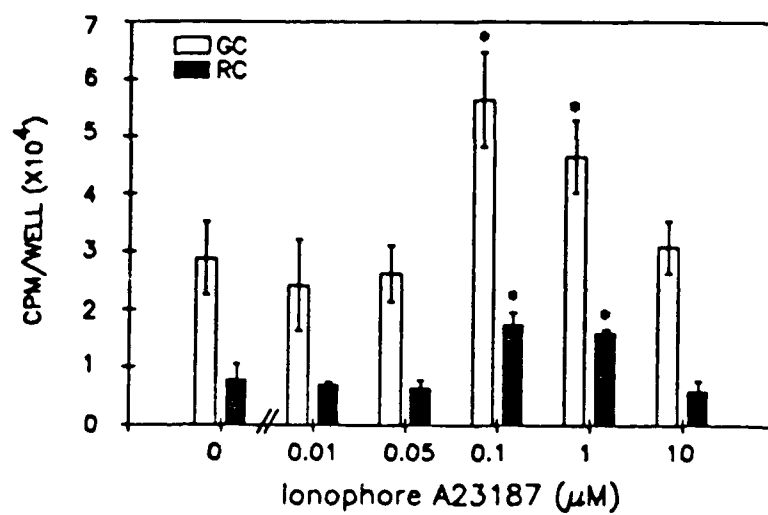
Figure 11

THE EFFECT OF 1,25-(OH)$_2$D$_3$ ON ^{45}Ca^{2+} INFLUX IN CONFLUENT, FOURTH
PASSAGE GROWTH ZONE CHONDROCYTES FROM 1-30 MINUTES.

Data represent the mean $\pm$ SEM of 6 samples. * Denotes p <
0.05 for sample v. control. Data shown are from a single
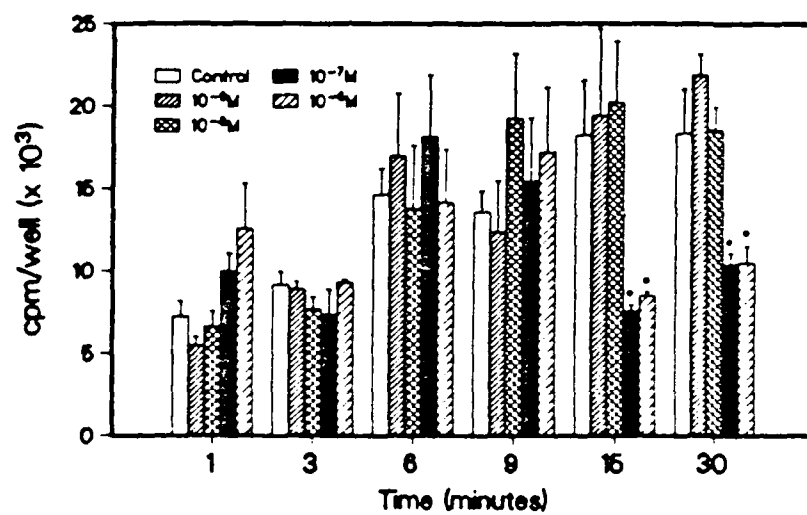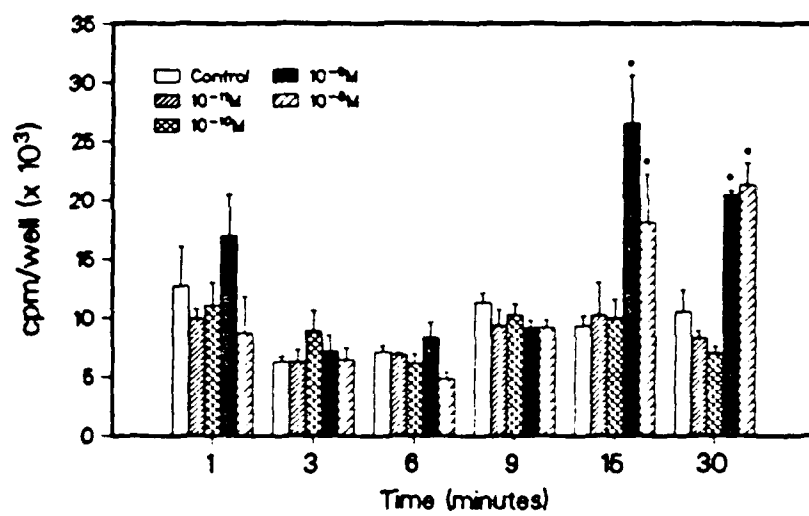experiment. Each experiment was repeated 3 times.

Legend (in plot):
- Control
- 10^{-12} M
- 10^{-10} M
- 10^{-9} M
- 10^{-6} M

Y-axis: cpm/well (x 10^3)

X-axis: Time (minutes)

at 10^{-8} M 1,25-$(OH)_2D_3$ and 1.9 fold at 10^{-9} M hormone. This was in contrast to the decrease in influx caused by the 24,25-$(OH)_2D_3$ metabolite at these time points in resting zone cells and differed from the ionophore effect seen only at 1 and 3 minutes. Other than the increase in $^{45}Ca^{2+}$ influx observed at 15 and 30 minutes, $^{45}Ca^{2+}$ content of the cells remained constant over test period whether or not exogenous hormone was added.

Calcium Efflux.

A23187. The effect of A23187 on calcium efflux was not determined since previous studies had failed to demonstrate an effect on bone cells (Dziak and Stern, 1976).

Vitamin D metabolites. Incubation with 24,25-$(OH)_2D_3$ inhibited $^{45}Ca^{2+}$ efflux from prelabelled resting zone chondrocytes at 10^{-7} M to 32% of control and at 10^{-6} M to 72% of control as soon as 1 minute (Figure 12). The effect was still present at 6 minutes but was gone by 15 minutes. Other than the rapid 24,25-$(OH)_2D_3$-induced inhibition of efflux at 10^{-6} to 10^{-7} M hormone, efflux of $^{45}Ca^{2+}$ from the cells remained constant throughout the incubation period.

In contrast, 1,25-$(OH)_2D_3$ promoted $^{45}Ca^{2+}$ efflux at 10^{-8} to 10^{-9} M at all time points tested (Figure 13). Peak stimulation occurred at 9 minutes (3.4 fold, 10^{-9} M). Efflux in the control cultures remained constant throughout the incubation at levels that were 1/3 to 1/2 that observed in the resting zone control cultures.

Influx and efflux in resting zone chondrocytes in response to 24,25-$(OH)_2D_3$ are compared in Figure 14. At early time

Figure 12

THE EFFECT OF 24,25-(OH)$_2$D$_3$ ON ^{45}Ca^{2+} EFFLUX IN CONFLUENT, FOURTH PASSAGE RESTING ZONE CHONDROCYTES FROM 1-60 MINUTES.

Data represent the mean $\pm$ SEM of 6 samples. * Denotes $p <$ 0.05 for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.
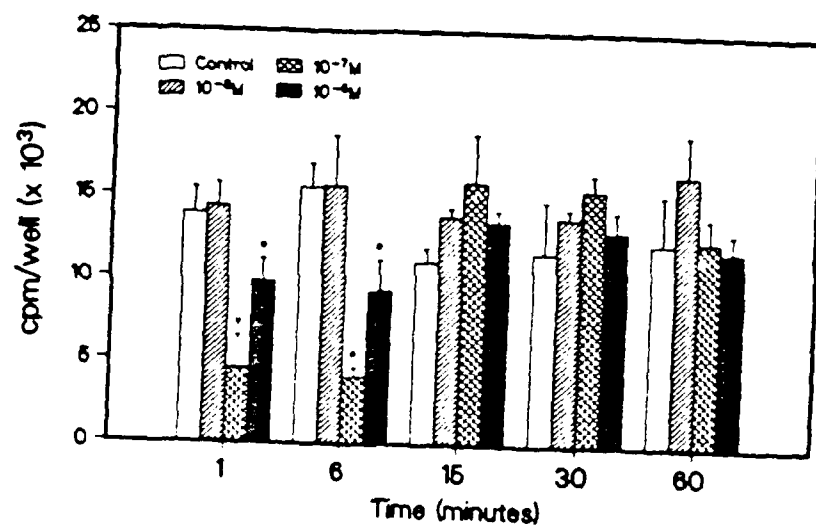
Figure 13

THE EFFECT OF 1,25-(OH)$_2$D$_3$ ON ^{45}Ca^{2+} EFFLUX IN CONFLUENT, FOURTH PASSAGE GROWTH ZONE CHONDROCYTES FROM 1-60 MINUTES.

Data represent the mean $\pm$ SEM of 6 samples. * Denotes $p <$ 0.05 for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.

Figure 14

THE EFFECT OF 24,25-(OH)$_2$D$_3$ ON ^{45}Ca^{2+} INFLUX AND EFFLUX IN CONFLUENT, FOURTH PASSAGE RESTING ZONE CHONDROCYTES FROM 1-60 MINUTES.

Data represent the mean ± SEM of 6 samples. * Denotes p < 0.05 for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.
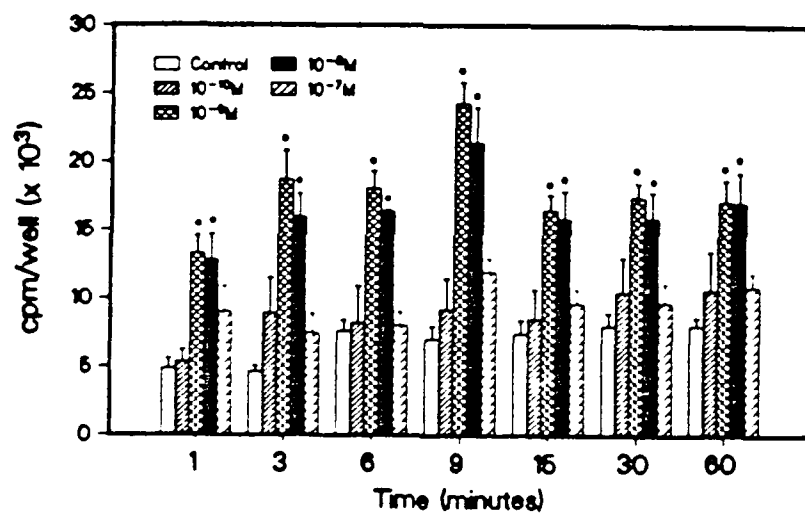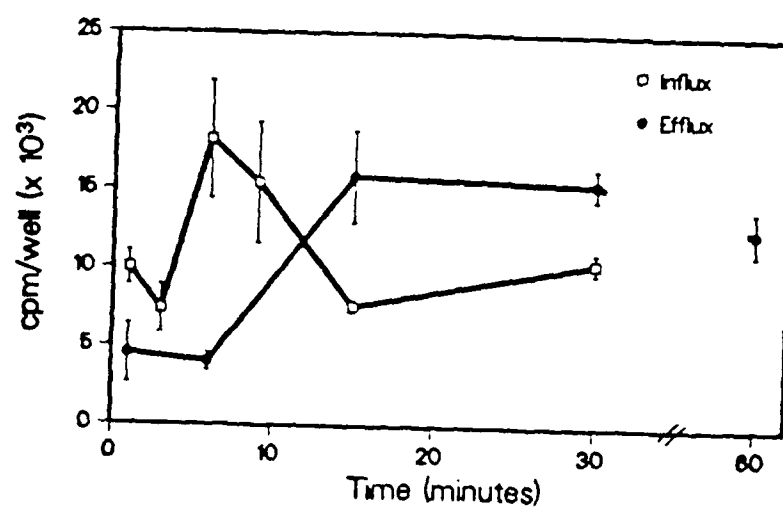
points there was an influx of $^{45}Ca^{2+}$, followed by a gradual decrease to beginning values. As influx began to fall, efflux began and plateaued at levels considerably higher than initial readings. The reverse was the case for growth zone chondrocytes in response to $1,25-(OH)_2D_3$ (Figure 15). At early time points efflux predominated. As efflux plateaued at initial values, influx of $^{45}Ca^{2+}$ was increased.

Discussion

Several investigators have shown that intestinal absorption of calcium is significantly elevated following administration of $1,25-(OH)_2D_3$ before any synthesis of transport proteins occurs (Wasserman and Fullmer, 1983; Fullmer et al., 1984; Yoshimoto and Norman, 1986; Nemere et al., 1986). One mechanism postulated for the rapid transport of $^{45}Ca^{2+}$ is that $1,25-(OH)_2D_3$ affects the morphological characteristics of biological membranes (Putkey et al., 1982), thereby facilitating trans-membrane ion movements. For example, Matsumoto et al. (1981) have shown that enhanced Ca^{2+} uptake in the intestine in response to $1,25-(OH)_2D_3$ is correlated with synthesis of phosphatidylcholine and increased incorporation of arachidonic acid. Similarly, Levy et al. (1987) have shown that $1,25-(OH)_2D_3$ stimulates phospholipid metabolism, preceding or coincident with Ca^{2+} flux. Studies in our laboratory indicate that phospholipase A_2 activity is stimulated by $1,25-(OH)_2D_3$ in growth zone chondrocytes but is inhibited by $24,25-(OH)_2D_3$ in

Figure 15

THE EFFECT OF $1,25-(OH)_2D_3$ ON $^{45}Ca^{2+}$ INFLUX AND EFFLUX IN CONFLUENT, FOURTH PASSAGE GROWTH ZONE CHONDROCYTES FROM 1-60 MINUTES.

Data represent the mean $\pm$ SEM of 6 samples. * Denotes p < 0.05 for sample v. control. Data shown are from a single experiment. Each experiment was repeated 3 times.
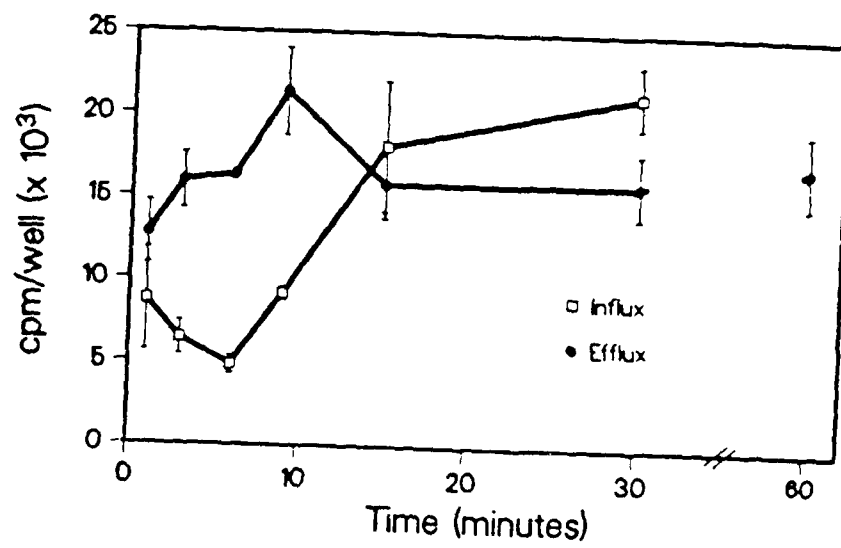
resting zone cells, suggesting that fluxes in Ca ion movement may be a mechanism by which these hormones work in cartilage cells as well.

Resultant changes in Ca^{2+} flux may have profound effects on cell metabolism (Kretsinger, 1980). Recent studies in our laboratory have shown that incubation of chondrocytes with the calcium ionophore A23187 at 0.1 uM for 3 minutes inhibited plasma membrane and matrix vesicle alkaline phosphatase specific activity (unpublished data), supporting the observations of Feher and Wasserman (1979) and Schiffl and Binswanger (1980) that a relationship exists between alkaline phosphatase activity and Ca^{2+} flux in non-mineralizing tissues (Haussler et al., 1970; Norman et al., 1970). However, although treatment with the ionophore could abolish the $1,25-(OH)_2D_3$ or $24,25-(OH)_2D_3$-stimulated alkaline phosphatase activity, it had no effect on the $1,25-(OH)_2D_3$-dependent stimulation of phospholipase A_2 in growth zone chondrocytes or on the $24,25-(OH)_2D_3$-dependent inhibition of phospholipase A_2 in resting zone chondrocytes (unpublished data). Therefore, we postulated that any regulation of these enzymes due to fluxes in Ca^{2+} movements must be different from those induced by A23187.

The present study confirms that $^{45}Ca^{2+}$ fluxes in response to A23187 are distinctly different from those produced by either vitamin D metabolite. A23187 produced a rapid transient increase in the rate of $^{45}Ca^{2+}$ uptake by both growth zone and resting zone chondrocytes similar to that demonstrated in fetal rat bone (Dziak and Stern, 1976). The effective concentration

of ionophore in the osteoblast cultures was 10^{-5} M v. 10^{-7} M in the chondrocyte cultures, suggesting that there may be differences in how A23187 interacts with the membranes of these two types of cells.

The magnitude of ionophore-induced $^{45}Ca^{2+}$ influx was much greater in growth zone chondrocytes than in resting zone chondrocytes, even within the same time frame. Since the mechanism by which A23187 functions in Ca^{2+} flux should be independent of cell type, this difference in response may indicate a difference in the characteristics or composition of the growth zone chondrocyte and resting zone chondrocyte membranes. Previous studies have shown that the membrane phospholipids of these chondrocytes do differ in composition (Boyan et al., 1988a). The difference in magnitude of response to ionophore may also reflect the possibility that only a subpopulation of resting zone cells respond with an increase in $^{45}Ca^{2+}$ flux whereas all or at least a larger subset of growth zone chondrocytes respond.

In our culture system, A23187, in concentrations that caused increased $^{45}Ca^{2+}$ influx, caused an inhibition of alkaline phosphatase specific activity (unpublished data). However, Schwartz et al. (1968b) have shown that vitamin D metabolites stimulate alkaline phosphatase specific activity in this chondrocyte model. This suggests that hormone-induced $^{45}Ca^{2+}$ flux is regulated differently from A23187-induced $^{45}Ca^{2+}$ flux. The difference may be in timing and/or mechanism of action.

1,25-$(OH)_2D_3$ induced Ca^{2+} influx in osteoblasts is blocked by verapamil and nifedipine but that induced by 24,25-$(OH)_2D_3$ is not affected by these inhibitors (Lieberherr, 1987). This suggests that the mechanism by which these metabolites work is different. This hypothesis is substantiated by the observation that, in chondrocyte cultures, 24,25-$(OH)_2D_3$ decreased influx at 15 and 30 minutes whereas 1,25-$(OH)_2D_3$ increased influx at the same time points. This observation correlates with the fact that 24,25-$(OH)_2D_3$ inhibits phospholipase A_2 in resting zone chondrocyte cultures whereas 1,25-$(OH)_2D_3$ stimulates activity of this enzyme in growth zone chondrocyte cultures (Schwartz and Boyan, 1988). Although the present study did not compare the effects of each metabolite on a single chondrocyte population, the differential effects of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ were measured on their primary target cell as defined by effects of hormone on Ca^{2+}-sensitive membrane enzymes.

In osteoblasts, 24,25-$(OH)_2D_3$ mobilizes Ca^{2+} from mitochondria and endoplasmic reticulum, 70% and 35% respectively, and by this mechanism elevates internal Ca^{2+} concentration (Lieberherr, 1987). Thus, the apparent decrease in influx induced by 24,25-$(OH)_2D_3$ in this study is probably indirect, via the influence of elevated cytosolic Ca^{2+} on influx. An increased release of cellular Ca^{2+} stores could also account for our measured decrease in $^{45}Ca^{2+}$ efflux via a simple dilution factor with unlabeled Ca^{2+}. Our data indicate that 24,25-$(OH)_2D_3$ inhibition of efflux ceases after six minutes, consistent with the time-span that 24,25-$(OH)_2D_3$ stimulates Ca^{2+}

release from osteoblast mitochondria and endoplasmic ret. culum (Lieberherr, 1987).

Since $1,25-(OH)_2D_3$ stimulated $^{45}Ca^{2+}$ efflux in growth zone chondrocytes at all the time points tested, any effect on $^{45}Ca^{2+}$ influx must be the result of the interaction of both influx and efflux. The increase in influx seen at 15 and 30 minutes in this study would result from the net change in total in-out of $^{45}Ca^{2+}$. The data show that the peak activation of efflux is at 9 minutes, which precedes the elevated influx seen at 15 minutes. Thus, influx detected by the $^{45}Ca^{2+}$ method used in this study may be related indirectly to the reduced level of efflux at 15 minutes. We do not know, by the methods used here, if there is increased influx in growth zone chondrocytes prior to 15 minutes. However, it seems this could be the case since there is no detectable reduction in influx seen at times when efflux is greatly elevated.

Influx and efflux of $^{45}Ca^{2+}$ are not correlated directly in either cell type in response to hormone. There is no significant effect of $24,25-(OH)_2D_3$ on influx in the resting zone cells at 1 and 6 minutes. However, efflux is significantly decreased. The reverse is true at 15 and 30 minutes when influx is reduced but there is no effect on efflux. In growth zone cells, $1,25-(OH)_2D_3$ increased influx at 15 and 30 minutes, but efflux was elevated at the early time points as well. The lack of 1 to 1 correlation between influx and efflux is also true for the cells incubated with ionophore. Further, atomic absorption spectroscopy data from both growth zone chondrocyte cultures and

resting zone cells (data not shown) show increased total Ca^{2+} amounts in the cells stimulated by ionophore.

One important observation of this study is that growth zone chondrocytes and resting zone chondrocytes handle calcium in a distinctly different manner, even under nonexperimental conditions. Influx of $^{45}Ca^{2+}$ in resting zone cells continued to increase over most of the incubation period, whereas efflux occurred at a steady rate. In contrast, in untreated growth zone cells, both influx and efflux remained constant, suggesting that under normal conditions these cells are more efficient at $^{45}Ca^{2+}$ efflux. This differential handling of cellular $^{45}Ca^{2+}$ is evident in the test cultures as well. The pattern of $^{45}Ca^{2+}$ accumulation and release is reminiscent of the behavior of these cells in the growth plate. Brighton and Hunt (1976) have shown that cells in the upper part of the growth plate load with Ca^{2+} whereas those cells in the lower hypertrophic and calcifying zones exhibit rapid efflux of Ca^{2+} from intracellular stores into the matrix.

Haussler et al. (1970) have proposed that vitamin D_3 might influence the molecular organization of targeted membranes and, as a result, influence Ca^{2+} permeability. Our data support this concept and suggest that the mechanism by which $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ influence membranes involves more than unregulated Ca^{2+} flux per se. The data also suggest that the two metabolites examined modulate $^{45}Ca^{2+}$ flux via different mechanisms. The action of $24,25-(OH)_2D_3$ could be explained by the cytosolic increase in Ca^{2+} released from the mitochondria

whereas the effect of $1,25-(OH)_2D_3$ is linked to its simultaneous effect on calcium influx and efflux.

Effect of $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ on Calcium Ion Fluxes in Costochondral Chondrocyte Cultures. Langston, G.G., Swain, L.D., Schwartz, Z., Del Toro, F., Gomez, R., Boyan, B.D. Calcified Tissue International, in press, 1989.

OVERALL DISCUSSION

Detailed discussions of the results of these investigations and their implications have been presented in Chapters I and II of this thesis. The following conclusions have been made from these results:

1. Alkaline phosphatase specific activity is higher in growth zone chondrocytes than in resting zone chondrocytes.

2. The effect of A23187 on alkaline phosphate specific activity is A23187 dose-dependent with inhibition at 0.1 uM in resting zone chondrocytes and at 0.1 uM and 1.0 uM in growth zone chondrocytes.

3. Alkaline phosphatase specific activity in matrix vesicles from resting zone chondrocytes was stimulated by incubation with 10^{-7} M 24,25-(OH)$_2$D$_3$; however, when 0.1 or 1.0 uM A23187 was added, the alkaline phosphatase specific activity was reduced to levels comparable to cells incubated with ionophore alone.

4. Combining A23187 with 1,25-(OH)$_2$D$_3$ in growth zone chondrocyte cultures had similar effects to those shown in resting zone chondrocytes. When growth zone chondrocytes that had been preincubated with 10^{-8} M 1,25-(OH)$_2$D$_3$ were challenged with 0.1 uM A23187, there was an inhibition of the hormone-stimulated alkaline phosphatase to levels achieved by A23187 alone.

5. Although A23187 had no statistically significant effect on phospholipase A$_2$ specific activity, a trend was noted which showed effects on phospholipase A$_2$ specific

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activity similar to the effects on alkaline phosphatase specific activity.

a. Efflux of $^{45}Ca^{2+}$ in growth zone chondrocytes was stimulated by 1,25-(OH)$_2$D$_3$ at 10^{-8} to 10^{-9} M at all time points tested with peak stimulation at 9 minutes.

b. $^{45}Ca^{2+}$ efflux remained constant throughout the incubation period for resting zone chondrocytes incubated with 10^{-8} M 24,25-(OH)$_2$D$_3$; however, at concentrations of 10^{-7} and and 10^{-6} M 24,25-(OH)$_2$D$_3$, efflux was inhibited at 1 and 6 minutes in resting zone chondrocytes.

c. The effect of 24,25-(OH)$_2$D$_3$ on $^{45}Ca^{2+}$ influx in resting zone chondrocytes was markedly different from that induced by A23187.

d. The effect of 1,25-(OH)$_2$D$_3$ on $^{45}Ca^{2+}$ influx in resting zone chondrocytes at 15 and 30 minutes was similar to that induced by A23187 at 1 and 3 minutes.

6. $^{45}Ca^{2+}$ influx of resting zone and growth zone chondrocyte cultures is increased within 1-3 minutes and decreased by 6 minutes after A23187 induction.

7. A23187-induced $^{45}Ca^{2+}$ flux differs from that produced by either vitamin D$_3$ metabolite studied.

8. A23187-induced $^{45}Ca^{2+}$ influx is much greater in growth zone chondrocytes than in resting zone chondrocytes.

9. In experiments studying effects of A23187 and vitamin D$_3$ metabolites on $^{45}Ca^{2+}$ influx and efflux, the control fluxes changed over time similar to changes in experimental cultures. These changes are presented graphically in

Figures 7, 8, 10, 11 and 12. Possible explanations for this are:

a. These are physiologic responses of the cells which are amplified to statistically significant levels by the addition of ionophore or hormones.

b. Changes in controls may reflect the response of the culture to the pH change precipitated by media replacement or to the temperature and pH changes following removal of the cultures from the incubator.

Each data point was compared by Student's t-test to its own negative control (no ionophore or vitamin D_3 metabolite treatment). However, changes in control cultures as a function of time were not analyzed statistically.

10. Resting zone chondrocytes and growth zone chondrocytes handle calcium differently. The change in $^{45}Ca^{2+}$ influx induced by $1,25-(OH)_2D_3$ in growth zone chondrocytes is opposite to that seen in resting zone chondrocytes stimulated by $24,25-(OH)_2D_3$.

11. Vitamin D_3 metabolites probably modulate $^{45}Ca^{2+}$ flux via different mechanisms.

Further studies should focus on the effects of changes in membrane phospholipid composition on this system. Additionally, the relationship between initial Ca^{2+} flux and subsequent protein synthesis, regulation and feedback need to be elucidated to better understand and therapeutically alter these responses.

Knowledge of the effects of $1,25-(OH)_2D_3$ and $24,25-(OH)_2D_3$ on chondrocytes at known stages of differentiation can eventually be used to characterize the effects of other growth factors on chondrogenesis. The significance of understanding the regulation of the mechanism of chondrogenesis will be realized by increased understanding of normal growth and of wound healing which occurs through endochondral ossification. Factors or hormones which regulate or alter these responses will be of great importance in designing therapy protocols for abnormal growth and to improve wound healing.

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VITA

Gregory Glenn Langston was born on May 11, 1952 to Lucille Taylor Langston and Julian Woodrow Langston, in Charleston, South Carolina. Following graduation from McClenaghan High School in Florence, South Carolina in June 1969, he attended Clemson University in Clemson, South Carolina. In 1973, he received his Bachelor of Science in Chemical Engineering degree from the Clemson University College of Engineering and was commissioned as a second lietuenant in the United States Air Force. After five years of service at the Air Force Armament Laboratory, Deputy for Systems Development and Deputy for Development Plans; Eglin Air Force Base, Florida, Captain Langston separated from the Air Force and entered the University of Florida College of Dentistry in August 1978 and received the degree of Doctor of Dental Medicine in December 1981. In July 1982, he re-entered the United States Air Force and completed a one year General Practice Residency at the USAF Regional Medical Center, Langley Air Force Base, Virginia in August 1983. After three years as a staff general dentist at Hahn Air Base, West Germany, Major Langston served one year as a staff general dentist at Kelly Air Force Base, San Antonio, Texas. In July 1987, he entered the Postdoctoral Periodontics program at the University of Texas Health Science Center in San Antonio in conjunction with Wilford Hall USAF Medical Center.

Publications

1) Langston, G.G., Swain, L.D., Schwartz, Z., Del Toro, F., Gomez, R. and Boyan, B.D.: Effect of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ on Calcium Ion Fluxes in Costochondral Chondrocyte Cultures. Cal. Tissue Int., in press, 1989.

2) Schwartz, Z., Langston, G.G., Swain, L.D. and Boyan, B.D.: Antagonistic Effects of A23187 on $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ Dependent Stimulation of Alkaline Phosphatase Activity in Costochondral Chondrocyte Cultures. Endocrinology, submitted for publication, 1989.

Abstracts

1) Langston, G., Schwartz, Z., Swain, L., Ramirez, V. and Boyan, B.: Effects of A23187 on membrane enzymes in cultured chondrocytes. AADR, 1989.

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